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Effects of A-beta immunotherapy and Omega-3 fatty acid administration in Alzheimer's transgenic mice

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Effects of A-beta Immunotherapy and Omega-3 Fatty Acid
Administration in Alzheimer's Transgenic Mice

by

Maren T. Jensen

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Alzheimer's Transgenic Mice

Maren T. Jensen

ABSTRACT

Major therapeutics against Alzheimer's disease (AD) are targeted towards reducing β -amyloid in the brain and improving cognitive performance. Transgenic mouse models of AD have become critical in the development of such therapeutics to protect against or treat AD. This dissertation examined the potential protective effects of both active A β immunotherapy and dietary omega-3 fatty acid administration to AD transgenic mice. First, immunization with A β_{1-42} from 2-16½ months of age provided protection against cognitive impairment in APP/PS1 transgenic mice well into older age. At both adult (4½-6 month) and aged (15-16½ month) test points, an extensive 6-week behavioral battery was administered that measured multiple sensorimotor and cognitive domains. A β immunotherapy either partially or completely protected APP/PS1 mice from impairment in reference learning/memory, working memory and/or recognition/identification at these test points. However, behavioral protection at the later test point occurred without any reduction in A β deposition within the brain. Therefore, the cognitive benefits of A β immunotherapy most likely involved neutralization or removal of A β oligomers from the brain. In addition to immunotherapy, this dissertation

also examined the behavioral and neurochemical effects of a high omega-3 (n-3) or high omega-6 fatty acid (n-6) diet to NT and AD transgenic (Tg+) mice from 2 through 9 months of age. The same 6-week behavioral test battery, as described above, was administered between 7½-9 months of age. In NT mice, dietary n-3 or n-6 fatty acids did not result in any beneficial effects on cognitive performance. In Tg+ mice, a high n-3 diet improved some, but not most, cognitive skills in comparison to standard-fed Tg+ mice; whereas a diet high in n-6 fatty acids did not lead to widespread deficits in learning or memory. In fact, there was no difference in overall performance on any behavioral task between Tg+ mice given a high n-3 or high n-6 diet. Administration of dietary fatty acids did not result in any significant changes in soluble or insoluble A β levels within the brains of Tg+ mice and plasma cytokine levels in Tg+ mice were largely unaffected. Notably, neither the high n-3 nor high n-6 diet increased cortical levels of n-3 or n-6 fatty acids, respectively, within Tg+ mice. However, NT mice on a high n-3 or high n-6 diet did show significant elevations in cortical n-3 or n-6 fatty acid levels, respectively, suggesting that Tg+ mice have a deficit in incorporation of dietary fatty acids in the brain. Collectively, these results show that life-long administration of active A β immunotherapy provides clear cognitive protection well into older age, whereas long-term dietary omega-3 fatty acid administration does not provide extensive cognitive benefit. Both studies underscore the value of using AD transgenic mice in determining the efficacy of prophylactics against AD.

Introduction

I. Alzheimer's Disease Background

Alzheimer's disease, first diagnosed by Alois Alzheimer in 1906, was not widely accepted as a disease affecting the elderly until the late 1960s. Within this period Alzheimer's disease (AD) arose as the most common form of senile dementia (Selkoe, 2001). The prevalence of AD is most common among people over 85 years of age (Munoz & Feldman, 2000). Primary clinical symptoms of AD include short-term and long-term memory loss, paranoia, delusions, decreased language ability and an overall cognitive impairment. The two hallmark pathologic features of AD include neuritic plaques and neurofibrillary tangles. Silver staining was first used in the early 1900s to indicate neurofibrillary tangles (NFTs) within neuronal cells. Following that discovery, in 1930 Divry used Congo Red staining to locate senile plaques composed of amyloid (Maccioni et al., 2001). In the 1960s Kidd and Terry first described in detail the composition of neuritic plaques and NFTs, which are composed of paired helical filaments (Selkoe, 2001). General risk factors for the disease include age, genetic mutations, and lifestyle influences such as high blood pressure, unhealthy diet, changes in hormones, and educational attainment/cognitive activity level.

II. AD Behavioral Deficits

During early AD, patients experience a loss of episodic, semantic and some implicit memory function (Becker et al., 1996; Salmon & Fennema-Notestine, 1996; Greene & Hodges, 1996). A loss of episodic memory appears similar to general amnesia so that patients are unable to learn new information; this denotes an impairment of storage and consolidation of new information (Becker et al., 1996). There is also an inability to shift information from short-term into long-term stores for later retrieval. Semantic memory is normally linked to long-term memory function of object, facts, concepts and words and their meanings, however in cases of early AD, there is a mild impairment in this type of memory function. A decrease in category fluency (generation of words from set categories, such as animals or fruits) is evident in early stages linked to a deficit of storage within the neural networks of the inferolateral areas of the temporal neocortex (Salmon & Fennema-Notestine, 1996). There is also an impairment of reading (comprehension of word meaning) due to the decrease in semantic memory function (Mathias, 1996). There are, however, no impairments in the lexical routes of reading, such as phonemic or syntactic skills at this early stage. Deficits in visual attention, extrapyramidal signs (bradykinesia, rigidity or tremors), ideomotor and ideational apraxias as well as nociceptive reflexes (snout and palmomental reflexes) are present in the early stages of AD (Coslett & Saffran, 1996; Kidron & Freedman, 1996). Despite discrepancies in language studies, language impairments have been shown to be evident in the early stages of AD (Becker et al., 1996).

Most of the above mentioned deficits progress as AD develops with addition of new cognitive impairments. From mild to moderate AD, 9-12% of subjects experience

hallucinations or delusions, with the range for severe AD between 40-60% (Schneider & Dagerman, 2004). It is proposed that these two symptoms of psychosis represent distinct subtypes of AD (Cook et al., 2003). AD subjects with psychosis undergo a more rapid cognitive deterioration than those with no psychotic symptoms (Cook et al., 2003; Schneider & Dagerman, 2004). Psychotic symptoms, representing hallucinations and delusions, are mainly a product of a frontal lobe dysfunction (Schneider & Dagerman, 2004). However, degeneration of the major brainstem aminergic nuclei results in symptoms of depression for moderate AD persons, affecting between 30-50% of AD cases. These subjects also have a relative preservation of the cholinergic system in comparison to non-depressive AD cases (Zubenko et al., 2003). Also present in moderate AD is an impairment of divided attention where attention can not be split between more than one task (Morris, 1996a). This is a role of executive function attributed to long-term memory where the deficit is nonglobal. There are also deficits in pyramidal signs related to changes in white matter (Kidron & Freedman, 1996). These include hyperactive jaw jerk reflex, extensor plantar responses, hyperreflexia and ankle clonus. Also present in about 10% of all moderate AD cases is motor impersistence (inability to sustain a voluntary movement) which is a deficit in sustained attention. More prominent in moderate AD is motor persistence (perseveration of movement) which is associated with the frontal system (Kidron & Freedman, 1996).

The late stages of AD show progression of cognitive impairment from the moderate stage of AD as well as impairment in procedural memory (Becker et al., 1996). Almost complete loss of both episodic and semantic memory occurs in the late stages of AD. In addition, late stage AD subjects that are severely impaired exhibit more of a

continuing loss of cognitive function compared to AD subjects with less impairment (Auer et al., 1994). Also evident are severe impairments of executive function, praxis and visuospatial behaviors. Severe deficits in language functioning can be seen by this stage including loss of fluency, echolalia, palilalia, non-verbal utterances and in some cases mutism (Boller et al., 2002). Seizures are also present in about 9.8-64% of all autopsy-verified late stage AD cases (Kidron & Freedman, 1996). Some cases of AD (20-41%) progress to a bedridden state, mostly due to the incidence of contractures that prevents subjects from ambulatory behaviors (Souren et al., 1995). Late stage AD patients can also exhibit a vegetative state, although because of differences in neurological evaluations there is a strong disagreement as to the incidence of a vegetative state in AD subjects (Volicer et al., 1997). Aside from additional symptoms of late stage AD, death is more common in severe AD cases due to pneumonia compared to non-demented control subjects (Beard et al., 1996). However in a retrospective study by Kammoun et al. (2000), there were no significant correlations between the incidence of AD and death due to bronchopneumonia compared to non-demented elderly control subjects; the authors did identify dementia as an underlying cause of death. In contrast, Hui et al. (2003) did show a significant correlation between increased mortality with increased cognitive decline in AD subjects over a four-year period, but the authors did not state the cause of mortality.

As previously mentioned a definitive diagnosis of AD is made only after death. This includes the presence of a large number of neurofibrillary tangle (NFT)-containing neurons and amyloid plaques within specific brain regions (Selkoe, 2001). Typically, NFTs are found primarily in neocortex and medial temporal structures including the

hippocampus, amygdala and parahippocampal gyri. Subcortical regions such as the thalamus, mamillary bodies, nucleus basalis of Meynert, substantia nigra and locus coeruleus also contain NFTs later in the disease process. In the AD brain, neuritic plaques appear first, and to their greatest extent in the cerebral cortex and hippocampus; subcortical structures such as the putamen of the thalamus, the locus coeruleus and the hypothalamus exhibit neuritic plaques to a lesser extent later in the disease process (Selkoe, 2001).

III. AD Pathology

Cerebral Atrophy, Neuronal Loss.

Alzheimer's disease results in an average of 7-8% decrease in total brain weight through atrophy (Morris, 1996a). Global atrophy is characterized by widened sulci, narrowed convolutions, decreased white matter and ventricular enlargement, specifically the lateral ventricles. The greatest loss of brain weight occurs in the cerebral hemispheres. Diffuse atrophy is associated with loss of cortex and hippocampal pyramidal neurons associated with the neocortex. The presence of NFTs in the cortex and hippocampus may account for the substantial cell loss in the nuclei that project to the neocortex, such as cholinergic neurons of the nucleus basalis of Meynert, noradrenergic neurons from the locus coeruleus, and serotonergic neurons of the raphe nuclei. There is a 40% decrease in the number of cells within the subiculum of the hippocampus (Morris, 1996c). Primary sensory and motor neurons, as well as thalamic projections experience minimal cell loss with AD. In normal aged individuals, there is no neuronal loss within layer II of the entorhinal cortex, with minimal loss within layer III (Trillo & Gonzalo,

1992). By contrast, in mild to severe AD cases there is a 50-90% loss of cells within the entorhinal layer II (Morrison & Hof, 1997). There is also a substantial loss of neurons within region CA1 of the hippocampus in mild to severe AD cases (Price et al., 2001). In addition, Fukutani et al. (2000) suggest that neuron loss in CA1 and entorhinal cortex is a direct result of neurofibrillary degeneration. Neuron loss within CA1 and subiculum of the hippocampus in severe AD has also been shown to correlate to formation of neurofibrillary tangles (Rossler et al., 2002). Specifically, pyramidal cells of the entorhinal cortex, CA1 and subiculum regions of the hippocampus are susceptible to neuronal degeneration and can lead to global disruption between association cortices (Morrison & Hof, 1997). In addition to neuronal loss in hippocampal regions, substantial loss occurs in the cortex. AD brains experience cortical atrophy in regions of the left hemisphere more than the right hemisphere (Gee et al., 2003; Thompson et al., 2003). Affected regions include the anterior temporal, posterolateral temporal and dorsolateral prefrontal regions (Gee et al., 2003), sparing the neurons of the sensorimotor cortex, occipital poles and cerebellum (Karas et al., 2003; Thompson et al., 2003). Overall, the total brain volume changes that occur with AD subjects are related to the accumulation of cortical NFTs (Silbert et al., 2003).

Measurements of regional cerebral blood flow (rCBF) by single photon emission computed tomography (SPECT) and positron emission tomography (PET) reveal decreases in brain functioning *in vivo*. SPECT and PET scans show maximal changes within the parietal and temporal brain regions and are consistent with neurodegeneration (Morris, 1996c). Correlations can be found between rCBF and neuropsychological functioning so that language dysfunction correlates with decreased left parietal and

temporal lobe rCBF. In PET studies there is a significant decrease in the resting activation of the parietal lobes of AD cases compared to normal controls. The greatest reductions are within regions that are impaired in early AD such as the middle and inferior temporal regions as well as angular and superior parietal gyri (Morris, 1996c). Implicated in the neurodegeneration of AD is the formation of amyloid plaques and development of intra-neuronal neurofibrillary tangles as discussed below.

Neuritic Plaques.

Amyloid precursor protein (APP) is a single transmembrane glycoprotein translated from chromosome 21. APP is a short-lived protein that is processed within the endoplasmic reticulum and subsequently modified through secretory pathways (Selkoe, 2001). Post-translational processing of APP within the endoplasmic reticulum includes glycosylation and proteolytic cleavage by specific enzymes (Selkoe, 2001; Turner et al., 2003b). APP is oriented so that the N-terminus is the extracellular domain and the C-terminus resides within the cytoplasm (Maccioni et al., 2001). There are three major isoforms of APP including residues ending with 695, 751 and 770. APP695 mainly occupies neurons, while APP751 and 770 are found in both neurons and non-neurons throughout the body. These two isoforms also contain a Kunitz class of serine protease inhibitors, also known as a KPI domain. Cleavage of APP occurs so that the derivatives are released into vesicle lumens and the extracellular spaces. There are three cleavage sites along the APP peptide. The first cleavage is provided by the α -secretase enzyme and releases a large soluble fragment (C83) with the COOH-terminal fragment within the membrane. The second cleavage site results from β -secretase to release a 99-residue

(C99) into the extracellular space or vesicle lumen. Cleavage by γ -secretase of the C99 fragment results in a smaller peptide, β -amyloid or $A\beta$, composed of exons 16 and 17 from the APP695 isoform (Selkoe, 2001; Ling et al., 2003). Cleavage by γ -secretase of the C83 peptide results in a smaller fragment named p3. APP is anterogradely transported to the axon terminals, the primary site for processing into $A\beta$. After APP is processed, left-over fragments of APP can undergo retrograde transport back to the cell body to be recycled. Recycling within the endosomes could lead to proteolytic cleavage by β - and γ -secretases to release the $A\beta$ peptide (Selkoe, 2001). Normal APP functioning includes cell adhesion, neuroprotection, neuroproliferative activation, intracellular and extracellular communication, as well as a cargo receptor for kinesin for microtubule transport and inhibition of serine proteases (Maccioni et al., 2001; Turner et al., 2003b). Deletion of the APP gene does not lead to any significant deleterious effects in *in vivo* experiments; however, cells cultured from APP deleted animals exhibit decreased neurite growth and nerve function (Selkoe, 2001).

In 1983 Allsop and colleagues identified neuritic plaques as containing a 40-42 amino acid peptide, known as the β -amyloid peptide (Turner et al., 2003b). $A\beta_{40}$ and $A\beta_{42}$ are both cleaved by similar processes utilizing β - and γ -secretases from the amyloid precursor protein. Diffuse plaques from APP cleavage result from accumulation of $A\beta_{42}$; these plaques are modestly fibrillar and nontoxic. Formation of diffuse plaques occurs mainly within association and limbic cortices (Roher et al., 2000). Neuritic plaque formation originates with α -helix structured monomers of either $A\beta_{40}$ or $A\beta_{42}$ peptides. Amyloidogenesis proceeds with deprotonation of specific side chain residues and

protonation of additional residues to cause a destabilization of the α -helix and conformational changes to a more stable A β dimer with a β -helical structure (Serpell, 2000). The dimer formation includes a hydrophobic core surrounded by hydrophilic residues. A ribbon-like protofilament is formed as a result of adjacent C-terminal binding between dimers to create a series of antiparallel β -sheets. Pairs of these β -sheets form a tubular cylinder within the center of each protofilament (Roher et al., 2000). Two types of protofilaments exist; the first includes a large diameter with periodic twists and the second type appears later in amyloidogenesis and forms no twists (Serpell, 2000). Fibril formation is a concentration dependant nucleation mechanism that utilizes 2-5 protofilaments. A hollow center is formed by hydrophobic residues through an intertwined helical structure with the hydrophilic N-terminus on the outside of the fibril; after the fibril is formed the N-terminus is degraded. Compact (dense) neuritic plaques, from aggregation of A β_{40} onto A β_{42} fibrils, are formed mainly in neocortex and hippocampus, as well as within cortical and leptomeningeal vessels (Roher et al., 2000). Dystrophic neurites, found in and around filamentous plaques, contain enlarged lysosomes, numerous mitochondria and paired helical filaments (Selkoe, 2001). Compact plaque cores are formed with a strong β -sheet conformation from simultaneous aggregation and disaggregation of A β fibrils. Microglia are associated with the plaque perimeter and commonly express CD45 and HLA-DR antigens. Reactive astrocytes form a ring surrounding the outside of the plaque with abundant glial filaments.

The presence of activated microglia and reactive astrocytes closely associated with mature amyloid plaques indicates that an inflammatory response has occurred to fibrillar A β deposits (Selkoe, 2001). Oskar Fisher first proposed that an inflammatory

process occurred in conjunction with amyloid fibrils in 1910. This proposal came without experimental evidence, however it is now known that fibrillar A β deposits are associated with a locally induced chronic inflammatory response (Fig. 1). The absence of T-cells and immunoglobulins from the A β plaque induced inflammation reaction suggests that this not a classical immune-mediated response (Eikelenboom et al., 2002). The inflammation associated with AD is due to the activation of local microglia from accumulation of fibrillar A β deposits. A β accumulation leads to the increased production of free radicals from microglia and peroxidative injury of proteins and lipids that can lead to selective neuronal dysfunction and cell death (Fig. 1) (Selkoe, 2001). Activated microglia secrete cytokines (TNF- α and IL-1 β) and neurotrophic factors (TGF- β 1) as well as generate free radicals (NO and superoxide) and fatty acid metabolites (eicosanoids and quilolinic acid) (Liu & Hong, 2002; Cotman et al., 1996). Binding of complement factor C1q to fibrillar A β deposits, with co-stimulation of serum amyloid P component (SAP) were shown to activate associated microglial clusters to initiate the classical complement cascade, as well as generate production of C5a (Eikelenboom et al., 2002; Cotman et al., 1996; Selkoe, 2001). Although microglia begin to appear during thioflavin positive staining, their major recruitment occurs after the generation of a proinflammatory anaphylactic peptide, C5a, is activated by fibrillar A β (Cotman et al., 1996). Reactive astrocytes are recruited by activated microglia, apparently in response to microglial cytokine secretion. Astrocytes are also the source of two inflammatory proteins which directly contribute to the formation of amyloid plaques (Selkoe, 2001; Potter et al., 2001)- α -antichymotrypsin (ACT) and apolipoprotein E (ApoE) (Fig. 1). The overexpression of IL-1, ACT and ApoE in specific brain regions showing

neuropathology in AD-affected individuals suggests a region-specific inflammatory cascade for AD (Potter et al., 2001). Together, ACT and ApoE have been described as amyloid promoters or “pathologic chaperones” from *in vitro* and *in vivo* studies, supporting their role in the AD inflammatory cascade (Sanan et al., 1994; Wisniewski et al., 1994; Bales et al., 1999). In addition, increased levels of ACT are found in AD patients’ serum and CSF (Sun et al., 2003) and associated with severity of AD dementia (Dekosky et al., 2003). In order to increase AD pathology, possible by inhibiting A β degrading enzymes, the ACT/A signal peptide variant increases the amount of mature glycosylated ACT for secretion. The level and location of APP processing to increase toxic C-terminal fragments can also result from an affect from ApoE. Nilsson et al. (2004) conclude that ACT and ApoE can act synergistically or independently to promote the development of mature amyloid plaques and diffuse A β deposits without affecting monomeric A β levels.

In addition to the inflammatory response, amyloid deposits also lead to a condition known as cerebral amyloid angiopathy (CAA) (Fig. 1). CAA is a deposition of amyloid within meningeal and cerebral arteries, arterioles, venules or capillaries and can occur in the absence of parenchymal A β deposits (Jellinger, 2002; Selkoe, 2001). The primary A β species present within blood vessels leading to CAA was determined to be A β ₄₀; recently, it was discovered that A β ₄₂ may also be present in amyloid angiopathy (Selkoe, 2001). CAA increases with age and results in thickened vessel walls through A β accumulation. Drainage channels and microglia take up the extracellular A β and deposit it in the vascular lumen to aid in clearance. Hyaline necrosis surrounding the amyloid

deposits within the blood vessel wall can rupture and lead to cerebral hemorrhaging. Cerebral hemorrhages linked to CAA occur primarily within frontoparietal cortex and parietal cortex with lesser amounts within temporal and occipital cortices, basal ganglia and cerebellum (Jellinger, 2002). Most subjects with AD do not have cerebral hemorrhage despite the large amounts of amyloid deposits within their blood vessels (Selkoe, 2001).

The β -amyloid peptide has many implications as previously described including formation of plaques, its role within the inflammatory process, and its link to CAA. In 1991, Hardy and Allsop first described the amyloid hypothesis. The hypothesis defined the root cause of AD as the overproduction and aggregation of $A\beta$ into senile plaques (Turner et al., 2003b). Multiple genetic risk factors such as an increase in the number of ApoE4 alleles, PS1, PS2 and APP mutations (see following sections) all increase the process of $A\beta$ deposition, leading to an increased risk for developing AD. Recent research has led to two changes for the initial amyloid hypothesis; the first is that oligomers and fibrils, in addition to diffuse/compact $A\beta$ deposits, contribute to the neuronal/cognitive dysfunction of AD, and the second is that changes in the activation of APP fragment molecules can also affect APP processing and $A\beta$ production (Turner et al., 2003b).

Neurofibrillary Tangles.

Neurofibrillary tangles are found within neurons of the hippocampus, entorhinal cortex, parahippocampal gyrus, amygdala and frontal, temporal, parietal and occipital cortices as well as certain subcortical nuclei (Selkoe, 2001; Maccioni et al., 2001). Some

transgenic mouse models suggest that A β stimulates NFT formation as discussed later in the “Animal Models” section (Fig. 1). Tau is a microtubule-associated protein that normally binds tubulin to aid in assembly of microtubule formation and stabilization within neurons (Mudher & Lovestone, 2002). Phosphorylation of tau by protein kinases cdk5 or GSK3 β causes it to dissociate from the microtubule and aggregate to form insoluble paired helical filaments (PHF) (Maccioni et al., 2001). Formation of PHF within neurons causes microtubule instability such that microtubules are eventually replaced by tangles (Mudher & Lovestone, 2002). Some studies indicate that NFTs, not amyloid plaque burden, show a high correlation with cognitive decline (Selkoe, 2001; Ohm et al., 2003). NFT formation occurs in brain regions that are implicated in memory. Within the cortex, tangles first form in the layer II of the entorhinal cortex prior to A β plaques (Ohm et al., 2003). Aside from a regional specificity, there is a cellular specificity in NFT formation. In some rare cases of AD only a small portion of cells are prone to form NFTs. In such cases, cortical pyramidal neurons can be “tangle poor” and instead can form Lewy bodies composed of α -synuclein proteins. Despite the few numbers of tangles that are present, these brain areas do elicit comparable amounts of A β plaques in the hippocampus with less deposition in the neocortex compared to AD brains that exhibit both NFTs and A β plaques (Terry et al., 1987).

A combination of A β plaques and NFTs could account for synaptic terminal loss in AD. Synaptic loss occurs at the terminal dendrite segments mainly within neocortical association areas, hippocampus, frontal lobe and temporal lobe. A decrease in synaptic density correlates highest with cognitive impairment (Coleman & Yao, 2003). Amyloid plaques have been found to alter synaptic integrity, but not to cause the loss of synapses.

PHF within the neuron disrupts axonal transport and results in starvation of the synaptic terminal and the eventual loss of the synapse (Scheff & Price, 2003). Studies have yet to show a direct correlation between synapse loss and either A β plaques or presence of NFTs.

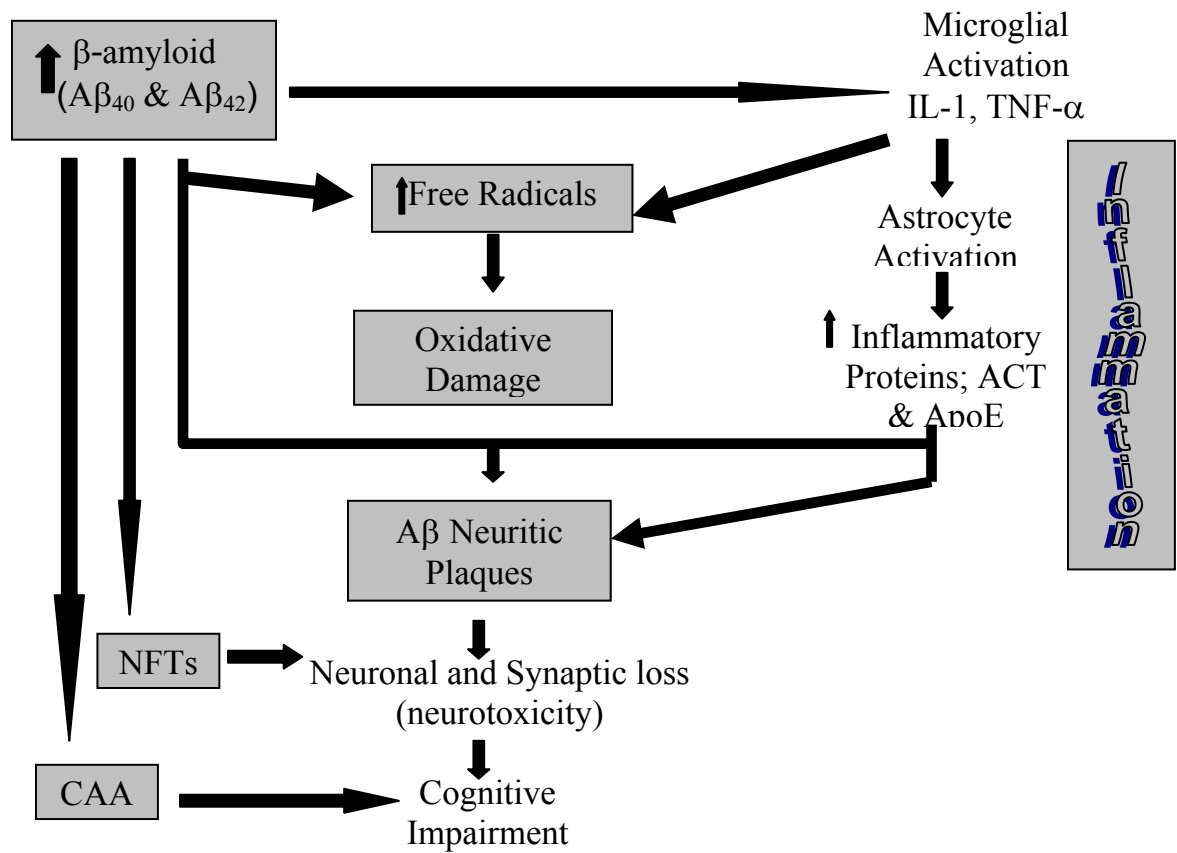


Figure 1. Flow diagram showing AD pathogenesis progressing to cognitive impairment.

IV. Genetics of AD

Familial Alzheimer's disease (FAD) is an autosomal dominant disease that accounts for 5-50% of all AD cases (Agnew, 1996; Selkoe, 2001). With little to no difference in phenotypic changes between FAD and sporadic AD (SAD) the main difference is the earlier age of onset for FAD (e.g. before 60 years of age), which has been linked to point mutations on chromosomes 1, 14 or 21.

The presenilin 2 gene from chromosome 1 can be affected by nine different mutations that increase the risk of developing AD; while chromosome 14 codes for the presenilin 1 (PS1) gene that can contain over 100 mutations (Marcon et al., 2004). Mutations of PS1 decrease the age of AD onset to the early 40's and 50's through a selective increase in γ -secretase cleavage of peptides C99 and C83; this mainly leads to an increase of $A\beta_{42}$ (Selkoe, 2001). Early-onset AD, from a PS1 deletion mutation, can result in verbal and visual memory impairment in addition to deficits in intellectual functions. These types of impairments were related to temporoparietal hypometabolism that is typically found in AD subjects (Verkkoniemi et al., 2004). One specific type of PS1 mutation, E280A, associates neuronal loss in the CA1 region and epileptic seizures of subjects with FAD (Velez-Pardo et al., 2004). The same mutation has been shown to cause an increase of $A\beta_{42}$, not $A\beta_{40}$, in FAD brains suggesting that PS1 can alter the cleavage of APP at the C-terminal end (Lemere et al., 1996).

AD risk can also be affected by mutations of the APP gene residence on chromosome 21. Down's syndrome patients (trisomy 21) have an overproduction of $A\beta_{40}$ and $A\beta_{42}$ as well as development of diffuse plaques by age 12 and neuritic dystrophy with NFTs in the 20's and 30's (Selkoe, 2001). The formation of NFTs in

Down's syndrome patients most likely occurs after the accumulation of extracellular A β deposits and inflammatory response (Mori et al., 2002). Specifically, Hirayama et al. (2003) proposed that NFTs form in Down's syndrome patients as a result of an axonal flow disturbance caused by the accumulation of A β_{43} within neurons of the cortex. Although most SAD and FAD cases have similar phenotypes, APP mutations can lead to an increased incidence of myoclonus, seizures and extrapyramidal signs (Agnew, 1996). There are up to 9 missense mutations that occur on the APP gene which facilitate cleavage to increase production of A β_{40} and A β_{42} . A point mutation at APP717 is referred to as the London mutation and typically results in an increase of A β_{42} (Selkoe, 2001). This mutation could result in a switch from valine to isoleucine in which case the onset occurs at approximately 50 years of age. Additional mutations from valine to glycine or valine to phenylalanine decrease the age of onset to within the 40's (Agnew, 1996). A double mutation at APP670/671 (Swedish mutation) increases the production of both A β_{40} and A β_{42} through increased β -secretase activation (Selkoe, 2001). The production of A β from the Swedish mutation is a result of competition between α - and β -secretase upon APP within Golgi-derived vesicles (Haas et al., 1995). Human subjects who possess the Swedish mutation form of AD exhibit reduced glucose metabolism within the temporal lobe which precedes cognitive impairment (Wahlund et al., 1999), and reduced regional cerebral blood flow within basal and lateral temporal lobes (Julin et al., 1998). Overall, although the SAD and above-mentioned FAD cases differ mainly in age of onset, there are also subtle differences in pathology as the disease progresses.

Apolipoprotein E (ApoE), which is involved in lipid trafficking, expresses three different alleles; ApoE2 is the least common, ApoE3 is the most common with

inheritance of ApoE4 giving rise to an increased risk for developing AD. Chromosome 19 contains the ApoE4 allele, which is over expressed in many AD cases. Inheriting 1 or 2 copies of the ApoE4 allele increases the risk for developing late onset AD in the 60's and 70's (Agnew, 1996; Selkoe, 2001). Studies show that the ApoE4 protein results in a higher A β plaque burden although there is no change in the amount of NFTs present. An increase in the number of A β fibrils could occur via a decrease of clearance or increased deposition of A β ₄₀ within the cerebral cortex and microvasculature (Selkoe, 2001). ApoE4 and ACT together promote deposition of monomeric and/or oligomeric A β filaments (Potter et al., 2001), in essence by acting as pathologic chaperones in binding to A β filaments. Glöckner et al. (1999) proposes that the action of ApoE primarily occurs at the early stages of AD. They found changes in hippocampal ApoE expression only within the brains of subjects during the beginning stage of AD (Glöckner et al., 1999).

V. Diagnosis of AD

The diagnosis of Alzheimer's disease occurs with complete assurance only after death by the presence of high numbers of amyloid plaques and NFTs within the brain. Prior to death, there are multiple cognitive exams designed to predict the presence of a cognitive impairment as an indicator for the development of AD. A diagnosis of AD follows three stages, the first is an initial screening into the patients' personal history; this establishes the subtype of the disease. The second stage involves a formal neuropsychological exam which determines the provisional diagnosis of the specific conditions of AD. The third stage includes a neurological exam and CT scan in order to exclude alternative forms of dementia not related to AD (Gray & Sala, 1996). Some of

the neurological screening methods used include the Mini-Mental State Exam (MMSE), which results in numbered scores administered to the patients. A score ranging from 0 to 17 implies severe cognitive impairment, whereas a score between 18 and 23 indicates mild to moderate impairment. Lastly, a score from 24 to 30 denotes an uncertain or absent degree of cognitive impairment (Crum et al., 1993). Additional cognitive exams include a brief telephone questionnaire or a paper and pencil skills test to screen for early signs of impairment, as well as multiple verbal memory tests. Some of these exams include the Cognitive Capacity Screening Examination, the Mental Status Questionnaire, the Short Portable Mental Status Questionnaire and the Dementia Rating Scale (Gray & Sala, 1996). Neurological screening exams are able to detect specific changes in memory function from early to moderate to severe forms of AD.

Additional screening methods include computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), functional MRI (fMRI), single photon emission computed tomography (SPECT) and positron emission tomography (PET). CT and MRI scans can detect anatomic changes within the brain, whereas MRS imaging measures metabolites as an indicator of brain function. For example, *N*-acetylaspartate (NAA) is a metabolite found within axons, choline (Cho) is in the myelin membrane and creatine (Cr) is involved in neuronal metabolic activity (Lee et al., 2003). PET and SPECT are additional forms of functional neuroimaging for glucose metabolism that can be measured within the brain.

In early AD, prior to cognitive decline, CT and MRI scans show cerebral atrophy and ventricular enlargement, including a progressive increase of cortical subarachnoid spaces. Some studies using CT scans show atrophy of the medial temporal lobes in AD

and volume loss of the hippocampus with MRI compared to non-demented controls (Lee et al., 2003). Glucose metabolic PET imaging with fluorodeoxyglucose (FDG) has the potential to detect early neocortical dysfunction before neuropsychological testing reveals dementia. As questionable dementia progresses toward mild AD, PET imaging detects abnormalities in the parietal lobe which exhibit asymmetrical differences in metabolism (Demetriades, 2002). PET imaging also shows a metabolic decrease in the posterior cingulate cortex, cinguloparietal transitional cortex, middle temporal cortex, inferior temporal cortex as well as angular and superior parietal gyri in early AD compared to age-matched controls (Demetriades, 2002). PET imaging can also detect amyloid deposition with labeled probes in early AD and presumably before cognitive symptoms are present. Multiple compounds have been tested as possible probes to bind A β within the brain such as a thioflavin-T analogue (Mathis et al., 2002), (18)F-labeled IMPY derivative (Cai et al., 2004), BF-108 (Suemoto et al., 2004), stilbene derivatives (Ono et al., 2003) and the Pittsburgh Compound-B (Klunk et al., 2004). Mathis et al. (2002) found that the thioflavin-T analogue, [(11)C]-labeled 6, has a high uptake within the brain of transgenic mice and resulted in labeled cerebral plaques and cerebrovascular amyloid deposits. The BF-108 compound was found to label senile plaques, neurofibrillary tangles and vessels laden with amyloid within temporal cortex and hippocampus of AD patients. BF-108 also labeled plaques within the brains of transgenic mice (Suemoto et al., 2004). In addition, the Pittsburgh Compound-B (PIB) had a high binding affinity within association cortex, most prominently within frontal, parietal and occipital cortices as well as the striatum. In areas such as the pons and cerebellum where there is minimal amyloid pathology, there was similar binding to that of non-AD control

subjects. The same study also found an inverse correlation between PIB retention within cortex and cerebral glucose metabolism (Klunk et al., 2004). Overall, the use of labeled probes by PET scanning to detect A β within the brain prior to cognitive impairment or in early AD is a useful tool for diagnosis and shows high affinity for amyloid plaques.

In moderate AD, MRS detects a decrease in NAA within temporoparietal, hippocampal and parahippocampal regions. A decrease in the NAA/Cr ratio of the occipital lobes confers a decrease in the involvement of functional neurons; a decrease within medial temporal lobes corresponds to cognitive decline (Lee et al., 2003). PET imaging studies show a global decrease in cerebral glucose metabolism, specifically the neocortical structures including the parietal, frontal and posterior temporal association cortices (Demetriades, 2002). A decreased metabolism of the parietotemporal cortex is a possible diagnosis for AD because of its high sensitivity and specificity to distinguish AD from other dementias. A substantial decrease is present in AD subjects of the resting activation of parietal lobes with the most amount of decrease within the occipital cortex (Morris, 1996c). PET studies also exhibit a hypometabolism within primary cortices, medial temporal cortex and posterior cortical regions (Demetriades, 2002). A bilateral hypometabolism of temporoparietal association cortex is present in AD and other dementias (Demetriades, 2002; Lee et al., 2003). PETs reveal a preservation of primary sensorimotor and visual cortices, cerebellum and striatum (Demetriades, 2002). Together, PET and SPECT studies show maximal changes within the parietal and temporal regions with a negative correlation between regional cerebral blood flow and neuropsychological functioning (Morris, 1996c). Language dysfunctions have been associated with left parietal temporal changes, whereas praxis disturbances correlate to

decreased regional cerebral blood flow within bilateral and temporal lobes (Morris, 1996b). In more severe cases of AD, there is a decreased metabolism within the frontal cortex (Demetriades, 2002; Lee et al., 2003).

For cases of FAD where subjects are homozygous for ApoE4, there is a significant decrease in the metabolic rate of glucose within posterior cingulate, prefrontal, temporal and parietal regions (Demetriades, 2002). In the same subjects prior to cognitive decline there is significant hypometabolism within the temporoparietal region (Lee et al., 2003). PET imaging of bitemporal and biparietal regions in FAD cases involving mutations of the APP gene show decreases in metabolism compared to age-matched controls (Demetriades, 2002).

Additional biomarkers for diagnosis of AD include cerebrospinal fluid (CSF) testing for the presence of total tau (t-tau), phosphorylated tau (p-tau) and A β ₁₋₄₂. Tau is a protein found within neuronal axons that has six different isoforms and 21 possible phosphorylation sites. The t-tau found within CSF of patients with MCI that progressed to AD is significantly increased compared to those with MCI that did not develop AD (Blennow & Hampel, 2003). The detection of t-tau has a sensitivity of 90% and specificity for distinguishing MCI from non-demented subjects of 100% (Blennow & Hampel, 2003; Hampel et al., 2003). Distinguishing early AD from normal aging using t-tau from the CSF reveals a sensitivity of 85% and a specificity of 75% (Hampel et al., 2003). In addition to Alzheimer's disease, increased CSF levels of t-tau are also found in other dementias including vascular dementia, frontotemporal dementia, lewy body dementia and semantic dementia (Blennow & Vanmachelen, 2003; Hampel et al., 2003). There are also studies showing a significant correlation between t-tau and cognitive

decline (Kanai et al., 1998; Nishimura et al., 1998), while additional studies show no correlation (Arai et al., 1995; Vigo-Pelfrey et al., 1995; Hulstaert et al., 1999).

Levels of p-tau are used to distinguish AD from control subjects through monoclonal antibodies specific to different epitopes. Overall, there is a mean sensitivity of 70% and a mean specificity of 94% with a mean increase in p-tau of 250% compared to age-matched controls (Blennow & Vanmachelen, 2003). At baseline, MCI patients have an increased level of p-tau versus control subjects (Buerger et al., 2002a; Andreasen et al., 2003). With progression from MCI to AD, levels of p-tau correlate with cognitive decline (Buerger et al., 2002a). Because levels of p-tau can distinguish MCI from non-demented control subjects, using CSF p-tau levels to diagnose AD prior to clinical dementia would improve treatment methods. CSF p-tau levels have also been inversely correlated with the age of onset for SAD and FAD subjects (Thaker et al., 2003) and positively correlated with the intensity of disease progression marked by a presence of ventricular widening during AD advancement (Wahlund & Blennow, 2003). CSF levels of p-tau correspond to the levels of p-tau within the brain and formation of tangles in AD; there are, however lower measurable levels of p-tau in vascular dementia, frontotemporal dementia and lewy body dementia as compared to levels present in AD (Blennow & Hampel, 2003). Using p-tau to accurately distinguish AD from other types of dementia is challenging. However, AD can be significantly distinguished from frontotemporal dementia by increased CSF p-tau of AD subjects (Buerger et al., 2002b). Hampel et al. (2004) also found that AD can significantly be distinguished from dementia with Lewy bodies by measuring CSF levels of p-tau. They conclude that because there is significant difference of CSF p-tau between two types of similar dementias, that p-tau must have

different roles within AD as compared to dementia with Lewy bodies (Hampel et al., 2004).

Overall, there is a decrease in the level of CSF $A\beta_{1-42}$ in AD as detected by ELISA with specific antibodies to $A\beta$. As previously discussed APP results in $A\beta$ by cleavage first by β -secretase releasing the C-terminus of APP into the extracellular space followed by cleavage by γ -secretase to release monomeric $A\beta$. Because of the close association between the CNS and CSF, $A\beta$ can flow into the CSF and levels can be measured for diagnosis. The mean sensitivity and specificity for $A\beta_{1-42}$ is 85% for distinguishing AD from age-matched controls (Blennow & Vanmachelen, 2003). There is also a strong association between decreased levels of $A\beta_{1-42}$ in CSF and increased number of plaques within hippocampus and neocortex (Blennow & Hampel, 2003). Some studies suggest that the decreased levels of CSF $A\beta$ are due to increased trapping of brain-produced $A\beta$ into amyloid plaques, although in Creutzfeldt-Jakob disease there are no deposited plaques despite a decrease in CSF $A\beta$ (Hampel et al., 2003).

Another indicator for developing AD is the inheritance of mutated forms of APP, PS2 or PS1 genes. APP, primarily exons 16 and 17, and PS2 mutations are detected from genomic DNA analysis of peripheral blood leukocytes amplified by polymerase chain reaction (PCR) and sequenced to give the exact gene. The PS1 mutations are commonly detected by extraction of total RNA from peripheral blood leukocytes for DNA amplification by a reverse transcriptase-PCR (RT-PCR) protocol. The entire PS1 gene is then sequenced to identify any possible mutations.

FAD from mutations of the APP gene are not prominently used as diagnostic markers for the detection of early-onset AD. Zekanowski et al. (2003) verified the presence of two APP mutations, T714A and V715A, in a population of patients with early-onset AD from Poland. They averaged the age of onset from either mutation around 55 years of age (Zekanowski et al., 2003). A second study found additional APP mutations in non-demented carriers, KN670/671ML and E693G (Almkvist et al., 2003). These APP mutant carriers exhibited no clinical abnormalities compared to non-carriers (Almkvist et al., 2003). Mutations from the PS2 gene are also not major contributors to FAD. However, one mutation of PS2, Q228L, decreases the age of onset to around 55 years of age (Zekanowski et al., 2003). Mutations of the PS1 gene are more common and carriers exhibit much of the same pathology from SAD subjects. This includes evidence of neuritic plaques, amyloid angiopathy, NFTs (Takao et al., 2002), diffuse plaques (Larner & du Plessis, 2003) and cortical degeneration mainly within the primary motor cortex (Miklossy et al., 2003). The average age of onset for some of the many PS1 mutations range from 25 years (Miklossy et al., 2003) to around 55 years (Zekanowski et al., 2003).

VI. Risk Factors for AD

The primary and most significant genetic risk factor for the development of late-onset AD is inheritance of at least one copy of the ApoE4 allele. Each ApoE4 allele inherited increases risk and decreases the age of onset for developing AD. About 80% of all FAD cases and 64% of SAD cases contain at least one copy of the ApoE4 allele,

whereas it is present in only 31% of control subjects (Corder et al., 1993). ApoE is synthesized within the brain and secreted by astrocytes. It plays a major role in lipid transport through the CNS (Olsen, 1998). However, the primary mechanism that ApoE4 affects to increase risk of AD is not through lipid transport, but most probably by affecting A β deposition. The binding of ApoE4 to A β ₄₂ increases A β deposition (Olsen, 1998) and density of neuritic plaques (Strittmatter & Roses, 1995; Nilsson et al., 2004). PDAPP transgenic mice, homozygous for murine ApoE4 had increased diffuse A β deposition within cerebral cortex compared to PDAPP ApoE knock-out (ApoE-KO) mice (Nilsson et al., 2004). The PDAPP + ApoE transgenic mice also had increased deposition of congophilic-positive compact A β plaques within cerebral cortex and hippocampus versus ApoE-KO mice. Together with the plaque pathology, ApoE mice exhibited significant memory deficits evident by spatial learning impairments and working memory impairments illustrated by the Morris Water maze and radial arm water maze tasks (Nilsson et al., 2004).

A second significant risk factor for the development of AD is high blood cholesterol levels. Increased total serum cholesterol level in mid- and late-life is positively associated with carrying the ApoE4 allele (Kivipelto et al., 2002). Accounting for this factor, a high level of total serum cholesterol is still a significant risk factor for developing MCI and AD (Kivipelto et al., 2002; Reitz et al., 2004). The mechanism of how cholesterol levels affect development of AD is unknown; however it is thought that A β production is indirectly increased by serum cholesterol levels, perhaps through stimulation of β -secretase processing of APP. Refolo et al. (2000) first determined that a high cholesterol diet results in increased amyloid deposition in the brains of AD

transgenic mice. Specifically, this study found increased amounts of β -C terminal fragments from APP cleavage resulting from β -secretase activity. Inversely, the increased β -secretase activity most likely resulted in a decrease of α -secretase activity which also led to the increased deposition of A β . Simons et al. (1998) also supported the theory that cholesterol affects β -secretase activity in cultured hippocampal neurons. In contradiction to the above studies, Burns et al. (2003) suggest that γ -secretase activity is highly affected by cholesterol accumulation in a mouse model of Niemann-Pick type C disease to increase production of A β_{40} and A β_{42} . Also, Wahrle et al. (2002) found an inhibition of γ -secretase activity in cultured brain cells with cholesterol depletion suggesting that γ -secretase activity is cholesterol-dependent. Also, treatments with cholesterol-lowering drugs as discussed in the next section have been shown to inhibit A β production through stimulation of α -secretase and decrease AD risk.

In addition to the affects of increased fat and decreased fish consumption on AD risk, reduced dietary intake of fruits and vegetables also contribute to the risk factor. To date, there have been no clinical longitudinal studies performed that administered DHA and/or EPA to test their protective effects against AD development; however some studies have administered fish oil supplements or a combination of omega-3 and omega-6 fatty acids to test their effects. Because of this, it is not known whether DHA and EPA, specifically, are conferring protection against dementia, or whether it is some additional aspect within the fish oil that results in beneficial effects. Specifically, clinical studies have shown that a high intake of fish, high in the polyunsaturated fatty acids DHA and EPA, is associated with a reduced risk for developing cognitive impairment, particularly

AD (Kalmijn et al., 1997a; Kalmijn et al., 1997b, Kalmijn et al., 2004). Kalmijn et al. (1997a) reported that an increased intake of total fat, saturated fat and cholesterol were all significantly associated with an increased risk for developing dementia, whereas increased fish consumption was the major factor associated with a reduced risk. Kalmijn et al. (1997b) also showed a significant correlation between increased fish consumption and decreased risk for developing dementia. The elderly subjects that developed dementia over the 8-year study were shown to have a higher intake of total fat, and the omega-6 fatty acid linoleic acid (LA) with a lower intake of fish, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) compared to those subjects that did not develop dementia. In contrast to the above studies, most recently Morris et al. (2005) reported a weak association between dietary intake of fish and rate of age-related cognitive decline. Instead, the authors suggested that overall fat consumption led to a greater risk of dementia. An additional study by Morris et al. (2003) also suggested that other fats play the primary role predicting the risk associated with developing AD. This study reported that saturated and *trans*-unsaturated fats were associated with developing AD, whereas n-3 fatty acids did not contribute or protect against risk of developing AD (Morris et al., 2003).

Aside from the possible involvement of nutrients from fish, dietary vitamin intake plays a role in risk for developing AD. A reduced dietary intake of vitamin B leads to increased plasma homocysteine levels (Selhub & Miller, 1992) and accumulation of DNA damage that contributes to AD risk (Kruman et al., 2002). Memory function is positively associated with intake of vitamins E, C and A (Solfrizzi et al., 2003; Meydani, 2003). Specifically, supplementation with vitamin E and vitamin C resulted in a

decreased risk of age-associated diseases including cognitive impairment (Meydani, 2003) and dementia (Haan & Wallace, 2004). Although some clinical studies have reported that vitamins E and C have no protective benefits against development of AD (Masaki et al., 2000; Laurin et al., 2004), additional clinical studies show a significant reduction of risk for developing AD with supplementation/intake of vitamins E and C (Morris et al., 1998; Engelhart et al., 2002b; Zandi et al., 2004). Animal studies involving non-transgenic rats (Joseph et al., 1998; Joseph et al., 1999) and mice (Shukitt-Hale et al., 1999) show improved cognitive performance with supplementation of various fruits and vegetables such as strawberries, blueberries or spinach. Supplementation with apple juice has also been shown to improve cognition and decreased oxidative stress in mice (Rogers et al., 2004). Overall, sporadic AD risk factors primarily include genetic predisposition (ApoE4 allele), high blood cholesterol (LDL) levels, high dietary intake of fats, and low dietary intake of fish, fruits and vegetables. Because SAD can not be accurately predicted, it is probably a combination of the above mentioned risk factors that all contribute to the development of AD.

VII. Treatment of AD

All of the US Food and Drug Administration approved treatments for Alzheimer's disease are aimed at providing relief from symptoms associated with cognitive decline. Current treatments include acetylcholine esterase inhibitors: donepezil (Aricept), galantamine (Reminyl), rivastigmine (Exelon), as well as the NMDA antagonist, memantine. Additional treatments have been tested in animal models, *in vitro* and

clinical trials including anti-inflammatory drugs, antioxidants and behavior modifications.

Tacrine was the first acetylcholine esterase inhibitor (AChEI) approved for AD symptoms. In a clinical trial tacrine relieved symptoms of hallucinations, anxiety, apathy and disinhibition in individuals with moderate dementia (Kaufer et al., 1996). A second clinical trial found improvements in language production, comprehension, word recognition and delusions in AD subjects with mild to moderate dementia (Raskind et al., 1997). Donepezil was approved as a noncompetitive and reversible AChEI for mild to moderate AD symptoms. It is metabolized in the liver by cytochrome P450 enzymes and uridine-diphosphate glucuronosyl transferase. Because of its long half-life (~ 70 hours) only one daily dose is needed (Scarpini et al., 2003). A clinical trial for 1 year including mild to moderate AD patients resulted in improved cognition and activities of daily living versus placebo-treated patients (Winblad et al., 2001). An additional study of moderate to severe AD subjects for 24 weeks showed that Donepezil improved cognition and decreased delusions, anxiety, disinhibition and irritability in these patients compared to placebo-treated subjects (Feldman et al., 2001). Galantamine was approved as a selective and reversible AChEI. It also functions as an allosteric ligand at nicotinic ACh receptors in order to increase presynaptic release of ACh and postsynaptic neurotransmission within pyramidal neurons. Galantamine is also metabolized in the liver by cytochrome P450 enzymes which results in a 5 hour half-life and therefore requires two daily doses for treatment (Scarpini et al., 2003). A clinical trial utilizing galantamine found it to decrease symptoms of anxiety, disinhibition, hallucinations and aberrant motor behaviors associated with mild to moderate AD, as well as improve cognition (Tariot et al., 2000).

Some therapies comparing the acetylcholine esterase inhibitors found differences in cognitive outcome. Jones et al. (2004) found a greater cognitive improvement with the use of donepezil versus galantamine whereas Wilcock et al. (2003) had opposite effects resulting in a significant cognitive effect of galantamine over donepezil. Despite differences from other studies, six months of treatment by the Cochrane Dementia Group with donepezil (Birks et al., 2002a), galantamine (Birks et al., 2002b) or rivastigmine (Olin et al., 2002) resulted in similar effects for all three drugs. Also, a similar study by Wilkinson et al. (2002) found no cognitive difference between donepezil and rivastigmine as treatments for patients with mild to moderate AD. All three acetylcholine esterase inhibitors show a stabilization of cognition through 6 months of treatment and as long as 36 months of treatment. At 6 months, donepezil (Tariot et al., 2001) and galantamine (Raskind et al., 2000; Patterson et al., 2004) were both found to maintain the improved cognitive performance in comparison to placebo-treated subjects. Following 12 months of treatment with galantamine (Raskind et al., 2000; Kurz et al., 2003) or rivastigmine (Doraiswamy et al., 2002) cognitive performance was maintained as compared to improved cognition after 6 months of treatment. Additional studies show maintenance of cognitive improvement through 18 months of treatment with donepezil (Matthews et al., 2000), 24 months of treatment with rivastigmine (Grossberg et al., 2004) and even 36 months of treatment with galantamine (Raskind et al., 2004).

Additional treatments include the use of statins and non steroidal anti-inflammatory drugs (NSAIDs). Statin use has been associated with a 39% (Zamrini et al., 2004) - 70% (Jick et al., 2000) decrease in the risk of developing AD. The mechanism by which statins decrease AD risk might be through a shift from γ - and β -

secretases to α -secretase to decrease A β production (DeKosky, 2003). *In vitro* studies show that β -secretase functions best in a high cholesterol environment so that a decrease in cholesterol leads to a decrease in BACE function. Statin therapy has been shown to lower cholesterol in cultured cells by blocking 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and thereby inhibiting A β production (Simons et al., 1998; Fassbender et al., 2001). Guinea pigs fed a diet supplemented with simvastatin showed a strong decrease of cerebral A β_{40} and A β_{42} levels from CSF and brain (Fassbender et al., 2001). AD transgenic mice fed a high cholesterol diet had increased β -amyloid deposition within the brains (Refolo et al., 2000). Additional mechanisms of statins may be that they remove A β from the brain or have a direct anti-inflammatory effect on AD through undetermined pathways (DeKosky, 2003). Because statins do not cross the blood-brain barrier their effects must be from a peripheral pathway that is not yet determined.

NSAIDs have been proposed to inhibit COX and therefore decrease the synthesis of prostaglandins, specifically PGE₂ and PGI₂ which are pro-inflammatory (Aisen, 2002a). Prostaglandins may potentiate glutamatergic transmission through NMDA receptors by inhibiting the reuptake of glutamate by astrocytes which leads to an excess of glutamate in the extracellular spaces (in 't Veld et al., 2002). In AD there is an increase of extracellular glutamate which leads to an increased activation of NMDA receptors and therefore an increase in intracellular calcium levels. The increased calcium could lead to neurotoxicity and neuronal cell death (Scarpini et al., 2003). The use of NSAIDs could inhibit this pathway. However, numerous studies have suggested that NSAIDs exert their protective effect independent of COX inhibition, by acting to inhibit

γ -secretase or its substrate. *In vitro*, the addition of ibuprofen, indomethacin or sulindac decreased the release of $A\beta_{42}$ within cultured cells that over express APP (Sascha et al., 2001). Along this line, a study involving cultured cells treated with either ibuprofen, indomethacin or sulindac sulphide found reduced $A\beta_{42}$ peptide independent of COX activity, most likely due to selective activity from γ -secretase to alter $A\beta$ production (Weggen et al., 2001). Additional work by Weggen and colleagues (2003) and Eriksen et al. (2003) also support the findings that NSAID activity can decrease levels of $A\beta_{42}$ within cultured cell lines and AD transgenic mouse models independent of COX inhibition. Weggen et al. (2001) found a significant reduction of $A\beta_{42}$ within the brains of APP transgenic mice with no change in $A\beta_{40}$ following treatment with ibuprofen. The authors suggest that this difference in $A\beta$ production is due a selective shift of γ -secretase activity. Similarly, administration of ibuprofen to APP transgenic mice resulted in a lower brain $A\beta_{42}$ levels (Sascha et al., 2001). NSAIDs could also inhibit inflammation by suppressing the expression of specific proinflammatory genes or by directly blocking the induction of interleukins 1 (IL-1), IL-1 β and possibly IL-6 (in 't Veld et al., 2002). A six month oral supplementation of ibuprofen to AD transgenic mice resulted in a significant decrease in $A\beta$ brain burden and microglial response (Lim et al., 2000). In addition, the NSAID NCX2216 has been shown to decrease $A\beta$ plaques in APP/PS1 mice more than ibuprofen or celecoxib (Jantzen et al., 2002).

Treatment with some NSAIDs resulted in no improvement in cognition in humans. A study by Van Gool et al. (2001) involving early AD subjects showed no cognitive or behavioral changes using the NSAID hydroxychloroquine versus the placebo

group. An additional study administering nimesulide to subjects with probable AD resulted in no significant effect on measures of cognition, activities of daily living or behavioral aspects compared to the placebo subjects (Aisen et al., 2002b). Aisen et al. (2003) also found no benefit to naproxen treatment with mild to moderate AD patients. A clinical trial for mild to moderate AD patients using indomethacin showed improved cognition with the drug compared to a decline of cognition in the placebo-treated group. However, indomethacin use had a low tolerance illustrated by adverse reactions to the drug and thus far there are no additional studies utilizing it (Rogers et al., 1993). Prednisone treatment to AD subjects also resulted in negative effects and decline in behavior with no change in cognition, after a 4-week period compared to placebo-treated subjects (Aisen et al., 2000).

In addition to anti-inflammatory drugs, antioxidants have been used as therapeutic treatment for AD. The aggregation of A β during AD progression can directly and indirectly induce oxidative stress. Regarding indirect induction of oxidative stress by A β , the interaction of A β with the receptor for advanced glycation end products (RAGE) in neuronal cell lines induces an increase of lipid peroxidation (Yan et al., 1998; Gasic-Milenkovic et al., 2003). Also, the interaction of A β with endothelial cells or blood vessels results in an increase in superoxide radicals leading to oxidative stress in the vasculature (Thomas et al., 1996). An additional mechanism for the indirect formation of oxidative stress by A β is through macrophages. Work by Klegeris et al. (1994) and El Khoury et al. (1996) both demonstrate the association between A β and macrophages to increase reactive oxygen species (ROS) such as nitric oxide. Regarding more direct actions, A β_{25-35} has been shown to directly generate ROS in the presence of O₂ (Hensley

et al., 1994). In addition, the aggregation of A β ₁₋₄₀ can lead to generation of free radicals that induce neurodegeneration through neuronal insults, such as lipoperoxidation or damage to intracellular proteins (Harris et al., 1995).

Ginkgo biloba has been defined as containing antioxidant and anti-inflammatory properties as well as being a memory enhancer. In a clinical trial involving patients with mild to severe AD there was a stabilization of cognitive and social function with 6 months to 1 year of Ginkgo biloba extract treatment (Le Bars et al., 1997). Mild to severe AD subjects showed improvements in cognition and daily behaviors following treatment from 3 months (Maurer et al., 1997) to 1 year (Le Bars et al., 2002) with high or low doses of the extract, EGb 761. In contrast to the above studies, mild to moderate AD patients supplemented for 24 weeks with high or low doses of EGb 761 did not show improved cognitive function (van Dongen et al., 2000; van Dongen et al., 2003). Other antioxidants, vitamins E and C, exert neuronal protection against oxidative damage and cell death. A Rotterdam study found a 34 – 43% decreased AD risk with supplements of vitamins C and E, respectively (Engelhart et al., 2002b). Vitamin E, α -tocopherol, has been associated with a decreased risk of AD in patients that were not carrying the ApoE allele (Morris et al., 2002). α -Tocopherol acts as an antioxidant by inhibiting lipid peroxidation within membranes as a free radical oxygen scavenger. It is the primary antioxidant candidate for AD and exerts significant beneficial effects on functional decline for patients with moderate to severe AD (Sano et al., 1997).

Aside from drugs and supplements, nonpharmacologic AD treatments include multiple types of behavioral modification. Few studies that try to improve cognitive and non-cognitive behaviors have been performed on AD patients. In 1989, Quayhagen &

Quayhagen administered a cognitive stimulation protocol including memory exercises, communication skills and problem-solving activities to AD patients. This resulted in maintenance of cognitive function across the treatment period, whereas the group that did not receive the treatment declined in cognition. More recently Sobel et al. (2001) tested the effect of the game of Bingo on subjects with AD versus AD patients that underwent daily physical activity. The AD subjects that played Bingo had improved performance of short-term memory, word retrieval and word recognition tasks. These alternatives can result in an increase of self-care skills as well as appropriate ambulation and socialization. Additional types of behavior modification include sensory and environmental intervention, structured activities and behavior therapy (Cohen-Mansfield, 2001).

VIII. Animal Models

Animal models have been used to aid in determining the functions of genes/proteins that were isolated from *in vitro* studies or to study the phenotypic results from gene changes, such as relocation of developmental transcription factors. In addition, transgenic animals from an overexpression or mutation in single or multiple genes are used to model human diseases and can therefore facilitate in determining possible preventions or treatments for such diseases. Most Alzheimer disease studies use mice as transgenic models. Even with the longer gestation and developmental period compared to invertebrates (*Drosophila* & *C. elegans*) commonly used as research models, physiologically mice are more closely related to humans and therefore provide a better foundation for a research model. Rats cannot generally be used to model human

disease and its effect on learning because of a lack of generation of developmental stem cell lines needed to generate rats lacking/over expressing a specific gene (Piccioto & Wickman, 1998). Due to this, mice are the most common and advantageous model used to study human neurodegenerative diseases such as Alzheimer's disease.

The generation of an AD transgenic animal requires the selection of the specific transgene cDNA to be transcribed downstream of a certain promoter sequence. The transgene DNA includes 1) the specific cDNA sequence that is to be over expressed, 2) the appropriate promoter specific for brain cells for neurodegenerative diseases that will create new mRNA and 3) a polyadenylation signal site that will stabilize the novel mRNA. The promoter is primarily important for determining the level of gene expression and the site and temporal pattern of expression.

The most common protocol for creation of transgenic animals is via pronuclear injection into a single-cell animal embryo. The fertilized eggs are removed from the female and injected with the transgenic cDNA. Multiple embryos (10-20) are then implanted into a pseudopregnant female foster mouse. A pseudopregnant female is generated by placing her, during a normal cycling period, with a vasectomized male. The transgene is either incorporated into the mouse genome or is degraded by endonucleases within the cell. If the transgene is incorporated early in development, all cells would inherit the transgene and be passed on to offspring. At weaning, 3 weeks of age, offspring are typically genotyped by polymerase chain reaction (PCR) for the presence of the transgene of interest. If the incorporation of the transgene is successful, roughly half of the offspring should inherit at least one copy of the gene and can pass it on to future generations. Alzheimer's disease research is performed with multiple lines of transgenic

mouse models as described in detail below in order to provide the most diversity for researching preventions and treatments.

PDAPP Transgenic Mice.

The PDAPP model is generated from a V717F mutation of the APP gene, also called the London mutation. Multiple studies have been done to characterize the pathology and behavior of the PDAPP model at various time points. Beginning at 3-4 months, PDAPP mice display an increase in synaptic density within cingulate, frontoparietal and parietal cortices (Dodart et al., 2000b). At this early age, there was also a substantial decrease in the size of the dorsal hippocampus as compared to the wild type mice. Mature A β deposits (plaques with dense cores) were first detected at 3-4 months within CA1 of the hippocampus, the medial part of the cingulate cortex and the corpus callosum level to the dorsal hippocampus. By 6-7 and 10-12 months, PDAPP mice display mature A β deposits with dense cores and significantly decreased dorsal hippocampus. Both age ranges displayed increased synaptic density within cingulate and parietal cortex, and decreased synaptic density in the dentate gyrus and septum. The 10-12 month mice also showed decreased synaptic density within region CA1 and the frontoparietal cortex (Dodart et al., 2000b).

One factor that plays a role in the amount of pathology present in this model is the number of transgenes present (heterozygous compared to homozygous). Between 3-4 months, all of the homozygous PDAPP mice exhibited some extent of mature A β deposits, whereas only half the heterozygous PDAPP mice revealed mature deposits (Dodart et al., 2000b). The 6-7 month homozygous mice had more A β deposits than the

10-12 month heterozygous mice. At the latter age, there was 3-4 times more A β deposits in homozygous than heterozygous PDAPP with the largest difference evident within the cortical regions (Dodart et al., 2000b).

In opposition to the above study by Dodart et al. (2000b), Games et al. (1995) measured plaque density and A β deposits and found no adverse pathology in heterozygous mice at 4-6 months of age. Only at 6-9 months of age were A β deposits detected within hippocampus, corpus callosum and cerebral cortex. By 9 months of age there was a decreased synaptic and dendritic density within the dentate gyrus. The study also reported that A β plaques from 9 month old heterozygous PDAPP mice were surrounded with GFAP positive reactive astrocytes and distorted neurites. In addition, the neocortex from these mice showed diffuse activation of microglia (Games et al., 1995).

Masliah et al. (1996) compared some of the AD-like pathology from 8-12 month old heterozygous PDAPP mice to tissue samples from AD brains. This study found similarities in amyloid deposition, dystrophic neurites and glial cell reactivity between the PDAPP mice and AD brains. The dense core structure of the PDAPP mouse plaques from hippocampus resembled the deposits found within frontal cortex of the AD brain. Similarities in dystrophic neurites and the dysfunction of their synaptic junctions were also seen. The synaptic junctions exhibited damage evident by enlargement of the nerve terminals and fewer synaptic vesicles. The majority of this damage was seen within plaques, not in the adjacent area surrounding the deposits. PDAPP mice also displayed glial cell reactivity (strong GFAP staining), similar to AD, associated with neuritic plaques and the neuropil of the neocortex and hippocampus. However, the transgenic

mice showed a stronger reactivity of astroglial cells and less microglial reaction compared to AD brains (Masliah et al., 1996). Overall, this study shows that the 8-12 month PDAPP mouse exhibits marked A β pathologic similarities to the AD brain.

In aged PDAPP mice, 16-17 months of age, Chen et al. (2000) found increased plaque density within multiple layers of the entorhinal cortex, the dentate gyrus, striatum and the molecular leucosum of CA1. At 18-21 months diffuse plaques were located within the hippocampus and cingulate, insular and entorhinal cortices (Chen et al., 2000).

Dodart et al. (1999) behaviorally tested heterozygous and homozygous PDAPP mice at 3, 6 and 9 months of age compared to age-matched non-transgenic littermates. Over all ages tested, PDAPP mice had increased activity and decreased rearings in a 50-minute open field task compared to wildtype (WT) mice; however there were no differences between any of the groups in a simple motor task indicating similar locomotive behaviors between all groups. Young (3-4 month old) PDAPP mice were found to be impaired in reference memory errors in the radial arm maze compared to WT mice. Working and reference memory impairment was correlated positively with increased synaptic density within cingulate and parietal cortices (Dodart et al., 2000b). In addition, reference memory impairment was positively associated with increased synaptic density in frontoparietal cortex and regions CA3 and dentate gyrus of the hippocampus. Over all 8 days of radial arm maze testing, the PDAPP mice did not show any learning effect, while the WT mice did show significant learning over all test days (Dodart et al., 1999). At this young age the PDAPP mice were not impaired in object recognition; however they had significantly fewer presses than wild type control mice in an operant memory bar task indicating memory impairment. Adult (6 months of age) and aged (9

months old) PDAPP mice also exhibited impairments in working and reference memory errors in the 8-arm radial maze task. As was the case for young PDAPP mice, there was also no learning effect evident compared to WT mice over the 8-day task for both 6 and 9 month ages. Adult and aged PDAPP mice were also impaired in object recognition showing a lower time spent exploring the novel object compared to the familiar object. Only at the 6 month age, there was a significant negative correlation between synaptic density in CA1 and the object recognition index (Dodart et al., 2000b)

Dodart et al. (2000a) also found an impairment of object recognition in 10-12 month old PDAPP and PDAPP+ apolipoprotein E Knock-Out (PDAPP/ApoE-KO) mice compared to age-matched WT and ApoE-KO littermates. This study also found a lack of habituation on day-1 of a two-day open field task for PDAPP and PDAPP/ApoE-KO mice; however WT and ApoE-KO mice did elicit habituation. The significance regarding the lack of habituation is suspect due to the fact that the activity of the PDAPP and PDAPP/ApoE-KO mice at the beginning of the trial equaled that of the WT and ApoE-KO mice at the end of the trial (i.e., after their significant habituation).

In contrast to Dodart, Chen et al. (2000) found normal object recognition in 6-9, 13-15 and 18-21 month old PDAPP mice compared to WT age-matched control mice. In a modified “working memory” version of the water maze task where mice were required to reach a criterion prior to changing locations of a submerged platform, 6-9 and 13-15 month old PDAPP mice exhibited a higher latency for each new platform location compared to wild type control mice indicating impairment of spatial acquisition memory. From 6 through 21 months, there was a significant negative correlation between the learning capacity in the modified water maze task and plaque burden (Chen et al., 2000).

Huitron-Resendiz et al. (2002) also found impairment of spatial memory; however it was evident only in aged PDAPP mice. Young (3-5 months of age) and aged (20-26 months old) mice were tested in a 12-day circular platform maze consisting of two trials per day. Young PDAPP mice performed similarly to WT mice initially and exhibited a significant increase in errors to locate the escape on the last block of testing only. In contrast, the aged PDAPP mice were significantly impaired to locate the escape on all trials as measured by errors made into non-escape holes. These results indicate a significant impairment of spatial memory that becomes slightly apparent at 3-5 months and prominent by 20-26 months of age in PDAPP mice.

Most recently, Nilsson et al. (2004) demonstrated the importance of inflammatory proteins, ApoE and ACT, within the PDAPP transgenic mouse lines. All four lines, PDAPP/ApoE/ACT, PDAPP/ApoE, PDAPP/ApoEKO/ACT and PDAPP/ApoEKO had similar amounts of soluble and membrane bound $A\beta_{1-40}$ and $A\beta_{1-42}$ in hippocampus and cerebral cortex at 2 months of age. At this age, all lines exhibited no sensorimotor, learning or memory deficits compared to non-transgenic mice. However, by 18 months of age, differences in pathology and cognition were evident between the different lines. PDAPP/ApoEKO/ACT mice had increased levels of $A\beta$ deposition within hippocampus and cerebral cortex compared to PDAPP/ApoEKO mice. Also, PDAPP/ApoE/ACT mice had increased deposition of compact $A\beta$ plaques in cerebral cortex and hippocampus due to an increase in plaque density compared to mice lacking ACT. In addition, both lines of ApoEKO mice showed no congophilic $A\beta$ deposition in cerebral cortex or hippocampus, indicating a primary role for ApoE to promote formation of compact plaques. Similar to the younger transgenic mice, none of the 18 month old PDAPP mice

showed any sensorimotor deficits compared to non-transgenic mice. However, all PDAPP/ApoE mice had increased latency (acquisition deficit) in the Morris water maze task compared to PDAPP/ApoEKO and non-transgenic mice. In addition, PDAPP/ApoE mice exhibited overall working and reference memory deficits in the radial arm water maze task compared to PDAPP/ApoEKO and non-transgenics. More specifically, PDAPP/ApoEKO/ACT showed deficits in working memory compared to PDAPP/ApoEKO and non-transgenic mice, indicating that ACT promotes the formation of amyloid, as described earlier, and cognitive decline in addition. This study also positively correlated reference memory from the radial arm water maze task to immunoreactive A β deposits and mature compact deposits in the hippocampus. Lastly, PDAPP/ApoEKO mice that showed diffuse A β in hippocampus had impaired working memory compared to PDAPP/ApoEKO that showed no diffuse A β , indicating the association between diffuse A β and working memory deficits. Overall, this study showed the importance of the PDAPP transgenic model interaction with ApoE and ACT genes (Nilsson et al., 2004). Using the same four PDAPP lines mentioned above, Leighty et al. (2003) found significant associations, between cognitive impairment in the Morris water maze, platform recognition task and the radial arm water maze task to A β deposition in the hippocampus and cerebral cortex in transgenic mice at 15-16 months of age. More specifically, working memory and recognition impairments were correlated with A β deposition in hippocampus and cerebral cortex. This study strongly supported the association between memory/ learning impairments to A β deposition within the PDAPP transgenic mouse (Leighty et al., 2003).

APPsw Transgenic Mice.

In addition to the PDAPP mutation, a commonly over expressed FAD double mutation at residues 670 and 671 of the APP gene has been termed the Swedish model, APPsw. Multiple APPsw models have been used with different backgrounds, providing a variety of results. APPsw model with an FVB/N background is highly vulnerable to neuronal cell death within the hippocampus (Mohajeri et al., 2004). Necrosis, apoptosis and astrogliosis within the hippocampus and cortex are also found within this mouse strain (Moechars et al., 1996). This mouse model also exhibits decreased glucose utilization and astrogliosis within the cerebellum (Hsiao et al., 1995). There were, however, no differences in levels of A β ₄₀ or A β ₄₂ compared to other FVB mixed backgrounds that do not exhibit the previously mentioned CNS disorders (Carlson et al., 1997). A predominant negative feature of mice with the FVB/N background is the prevalence of retinal degeneration that occurs around post-natal day 5 for transgenic mice and post-natal day 9 for non-transgenic mice (Vinores et al., 2003); therefore cognitive evaluation of this strain becomes difficult. With this in mind, FVB/N mice are spontaneously hyperactive and aggressive; in addition, these mice exhibit poor performance in spatial and non-spatial tasks (Voikar et al., 2001; Mineur & Crusio, 2002). Additional behavioral studies suggest that the prevalence of premature death, neophobia, aggression and seizures are dependent on the level of APP expression (Hsiao et al., 1995; Carlson et al., 1997). Neophobia has been associated with thigmotaxis, agitation and tremulousness as early as one month of age (Carlson et al., 1997).

A second model of the APPsw mouse is the APP23 model, generated by insertion of the mutated gene into an *XhoI* site of an expression cassette containing the murine

Thy-1.2 gene (Sturchler-Pierrat et al., 1997). At 6 months of age, this line shows rare A β deposits, but at 24 months there is a substantial deposition of A β plaques, with dense cores, including an increase in size and number of plaques associated with an increase of age. Deposits occupied regions of the thalamus, olfactory nucleus, caudate putamen, neocortex and hippocampus; these regions also contained a massive glial response. A β deposits were associated with a decrease in the number of adjacent cell bodies in hippocampal pyramidal cells and were also surrounded by dystrophic neurites (Sturchler-Pierrat et al., 1997). Robust plaque deposits were present even at 14-18 months of age throughout the neocortex and hippocampus of heterozygous and homozygous APP23 mice (Calhoun et al., 1998). The plaque load within region CA1 of the hippocampus inversely correlated with the number of pyramidal neurons within this area constituting a significant neuronal loss compared to non-transgenic control mice. There was, however no significant neuron loss within the neocortex despite the high load of plaques present. Amyloid was also detected within vessels of the meningeal, neocortical and thalamic vasculature at the same age of 14-18 months (Calhoun et al., 1998).

Spatial memory deficits exhibited by the Morris water maze task preceded the formation of plaque deposition within APP23 heterozygous mice (Van Dam et al., 2003). At 3 and 6 months of age, these mice showed significant learning and memory deficits in Morris water maze acquisition and retention; however significant formation of plaques occurred first at 6 months (Van Dam et al., 2003).

In agreement with Van Dam, 3, 18 and 25 month old APP23 mice were found to be impaired in the Morris water maze, as exhibited by increased latency and distance swum to the hidden platform (Kelly et al., 2003). Also at all ages, APP23 mice had

fewer annulus crossings in the memory retention trial compared to the non-transgenic mice; however, only at 3 and 18 months did the APP23 mice have a lesser amount of time spent in the former platform-containing quadrant versus age-matched non-transgenic mice in this memory retention trial. Because all mice performed similarly to age-matched non-transgenic mice in the visible platform version of the water maze, the authors suggested that the spatial impairment of the APP23 mice was not due to sensory, motor or motivational alterations.

As opposed to the above findings, Lalonde et al. (2002) found impairment of acquisition in the Morris water maze of 16 month old APP23 mice; however, there was no impairment in the probe trial (memory retention). These mice also exhibited hypoactivity in contrast to the hyperactivity found in mice ranging from 6 weeks to 6 months of age (Van Dam et al., 2003).

An additional model of the APPsw mouse was created with a C57BL/6 X C57BL/6/SJL background, the Tg2576 mouse model. Multiple studies have focused on analyzing various markers to characterize the Tg2576 mouse such as A β plaque deposition, and inflammatory, synaptic and oxidative markers as indicators of an AD-like pathology within a mouse model. Hsiao et al. (1996) first classified the Tg2576 APP mouse by analyzing brain A β_{1-40} and A β_{1-42} levels as well as senile and diffuse plaques within 2-8 month and 11-13 month old transgenic mice. As early as 2 months of age, Tg2576 APP mice showed increased neurochemical levels of A β_{1-40} , with a 5-fold increase by 11 to 13 months of age, and A β_{1-42} with a 14-fold increase by 11 to 13 months. In addition to brain levels of A β , amyloid deposits were seen in the older (11-13 month) transgenic mice within the frontal, temporal and entorhinal cortices, in addition to

the hippocampus and cerebellum. Associated with these A β deposits were immunoreactive astrocytes and dystrophic neurites (Hsiao et al., 1996).

In agreement with Hsiao et al. (1996), Pratico et al. (2001) showed that the Tg2576 APP mice exhibit A β ₄₀ and A β ₄₂ at low levels in the brain from 4-8 months with high levels seen at 12 months, progressing into 18 months of age within the cerebral cortex and hippocampus. Scattered A β deposits within cerebral cortex and hippocampus were not evident until 12 months of age with more abundant deposits seen in the neocortex and hippocampus at 18 months (Pratico et al., 2001).

In conjunction with Pratico, Benzing et al. (1999) found moderate A β deposits within 12 month old Tg2576 mice with considerable deposition occurring by 18 months of age. However, the A β deposition was predominately found within temporal and cingulate cortices and to a lesser extent within frontoparietal cortex and hippocampus. This study also examined the presence of astrocytes and microglial cells and their association with plaque deposition. Astrocytes were mainly localized near fibrillar deposits typically on the periphery of the plaque; microglial positive activity was detected around A β deposits at 12 and 18 months. In conjunction with the absence of A β deposits at 3 months of age, there was also no detection of astrocyte or microglial activity. The presence of the inflammatory markers, CD-45 and MAC-1 were positively associated with microglial activation and fibrillar A β deposits at 12 and 18 months, whereas IL-6 immunoreactivity was associated with astrocyte activation near fibrillar A β deposits also at 12 and 18 months within the Tg2576 mouse (Benzing et al., 1999).

In contrast to the above studies, Mehlhorn et al. (2000) did not detect any deposition of fibrillar A β plaques within cortex of Tg25765 mice through 13 months of age. At 14 months, a low burden of A β deposits was detected within cerebral cortex and hippocampus with the presence of reactive astrocytes detected around the plaques. This study also detected IL-1 β immunoreactive cells around A β plaques that co-localized with the astrocyte activation at 14 months (Mehlhorn et al., 2000).

Irizarry et al. (1997) detected A β deposition in Tg2576 mice at 16 months of age within the cingulate cortex, molecular layer of the dentate gyrus, entorhinal cortex and region CA1 of the hippocampus. At the subcortical levels, rare deposits were evident in the internal capsule and the basal ganglia at this late age. In conjunction with the above references, positive astrocyte activation was associated with neuritic plaques. This study also looked at neuronal pathology in reference to the progression of AD. They found that dystrophic neurites were found surrounding and within A β deposits, however there was no difference in the actual number of neurons within region CA1 compared to non-transgenics, despite the amount of A β deposits within that area. This study also showed no difference in synaptophysin staining (a glycoprotein located in neuronal synapses and vesicles) of the outer, middle or inner molecular layers of the dentate gyrus between Tg2576 and non-transgenic animals (Irizarry et al., 1997).

Although there were no differences seen in synaptophysin staining at 14 months by Irizarry et al. (1997), King and Arendash (2002b) noted strong staining within interior cortex and hippocampus of 19 month old Tg2576 APP mice compared to non-transgenic mice, but not at any of the younger ages. The strongest staining occurred in the plaque periphery, with minimal staining present within the core of plaques. Tg2576 mice

exhibited a linear plateau of synaptophysin staining from 3 to 19 months, whereas the non-transgenics have a decreasing trend up to 19 months creating the significant difference between the genotypes. Thus, synaptophysin was higher within the neocortex of Tg2576 mice at 19 months compared to non-transgenic mice. As well, the outer molecular layer and polymorphic layer of the dentate gyrus had increased synaptophysin staining in the 19 month APP mice versus non-transgenics (King & Arendash, 2002b).

In addition to the pathologic markers that are exhibited in the Tg2576 APP model, changes occur in oxidative stress markers as well. Pratico et al. (2001) isolated and quantified the presence of isoprostanes (sensitive markers of lipid peroxidation and oxidative stress) within urine, plasma and brain tissue of Tg2576 APP mice from 4 to 18 months of age. Human AD subjects exhibit increased isoprostane (IsoP) levels within urine, plasma and CSF, therefore this oxidative marker could be used as a useful peripheral indicator for AD. After 6 months of age, APP mice show significant elevations of IsoP levels within the urine with a maximum plateau occurring after 12 months of age compared to non-transgenic control mice. Plasma levels of IsoPs are significantly increased after 8 months in APP mice versus non-transgenic animals. As a general reference, there was no difference found within the non-transgenics at any age within urine or plasma IsoP levels. Prior to 8 months there was no difference in brain levels of IsoPs, specifically cerebral cortex and hippocampus. At 8 months and older, cerebral cortex and hippocampus exhibit increased levels of IsoPs compared to non-transgenics with no difference at any age within the cerebellum (Pratico et al., 2001).

In agreement with Pratico, Smith et al. (1998) found increased oxidative stress markers in 13-25 month old Tg2576 APP mice. This study specifically analyzed

hemeoxygenase-1 (HO-1) and HNE (lipid peroxidation marker). Both HO-1 and HNE were found to be associated with the peri-plaque regions and neuronal cell bodies that were distant from A β deposition. Their presence at the distant locations indicates a global increase of oxidative stress. However, the detection of reactive oxygen species (ROS) was only found to be present at the sites of A β deposition (Smith et al., 1998). In a similar age of mice, Pappolla et al. (1998) detected an increase of superoxide dismutase (SOD) by 21-25 months of age. The presence of SOD overlaps with the evidence of dystrophic neurites and A β plaque deposition. The authors suggest that the overlap between SOD detection and A β deposition supports the idea that A β is neurotoxic and that toxicity is mediated by free radicals (Pappolla et al., 1998).

Aside from the plethora of pathologic evidence used to characterize this mouse model, behavioral analyses of learning and memory are also present to further support the similarities between the progression of AD in humans and in mouse models. At 3 months of age, the Tg2576 mouse was found not to be impaired in sensorimotor tasks, Y-maze alternation or entries, Morris water maze or visible platform (Holcomb et al., 1999). However by 6 and 9 months of age, they exhibit a significant decrease in Y-maze alternation compared to non-transgenic mice indicating a deficit in basic memory function. This study found no impairment in visible platform or Morris water maze acquisition or retention even at 9 months of age. Whereas Holcomb et al. (1999) found no impairment in visible platform at 9 months, King and Arendash (2002a) found a significant increase in latency at 9, 14 and 19 months for the Tg2576 mouse compared to non-transgenic mice.

In agreement with Holcomb, King and Arendash (2002a) found no impairment at 3 months age in Morris water maze, visible platform and Y-maze entries, as well as circular platform. They did, however, find impairment in Y-maze alternation, an increase of open field activity and poor balance beam performance at this early age. This study behaviorally tested mice from 3-19 months of age which resulted in overall impairment in open field activity, balance beam performance, string agility and Y-maze alternation in comparison to age-matched non-transgenic mice (King & Arendash, 2002a).

In contrast to King and Arendash, Hsiao et al. (1996) did not observe an impairment of Y-maze alternations at 3 months, but did see impairment at 10 months of age within Tg2576 mice. Also at 10 months of age the mice exhibited acquisition deficits, evident by increased latencies in the Morris water maze as compared to non-transgenic control mice. Mice between 9 to 15 months had a significant impairment of memory retention also within the Morris water maze. Overall, the authors suggest an association between the appearance of A β ₄₀ and A β ₄₂ neurochemically within the brains of 2 month old APP mice that progressively increased through 13 months to the progression of learning deficits that were observed in these ages (Hsiao et al., 1996).

Westerman et al. (2002) also found significant impairment of memory retention within the probe trial of the Morris water maze in groups of mice ranging from 6 to 25 months of age, however no impairment was evident in very young mice (4-5 months old). The presence of insoluble A β was detected in all mice over 10 months old; therefore the authors suggested an association between the presence of A β _{insol} and the occurrence of memory impairments within this mouse model (Westerman et al., 2002).

APP/PS1 Transgenic Mice.

The last model of Alzheimer's disease reviewed here is a double transgenic mouse involving the APP^{sw} mutation and a missense mutation of the presenilin 1 gene. In general, APP/PS1 mice exhibit an accelerated amyloid deposition compared to the single transgenic APP mouse. Some studies even suggest that deposition begins to occur as early as 3 months of age. Holcomb et al. (1998) detected the presence of a modest amount of compact A β deposition between 12-16 weeks of age. By 24-32 weeks of age the deposition was increased and the plaques became surrounded by reactive astrocytes (Holcomb et al., 1998). In agreement with Holcomb, Takeuchi et al. (2000) detected A β deposition in the double transgenic model at 3 months of age. Specifically, plaques were found in cingulate, superior frontal and parietal neocortices with a lesser amount detected within the hippocampus. From this age, the deposition progressed to include larger compact plaques scattered throughout the cortex and small diffuse plaques displayed by 6 months of age. By 9 to 12 months, larger dense plaques and small diffuse plaques had increased in number and were found within the neuropil of the entire neocortex. Despite the increased number of plaques found by 12 months, Takeuchi et al. (2000) did not see any changes in the number of neurons present in region CA1 or in synaptophysin immunoreactivity in the molecular layer of the dentate gyrus and CA1 compared to age-matched non-transgenic and single APP transgenic mice.

In contrast to the above studies, Gordon et al. (2002) did not detect any A β deposition at 3 months of age in APP/PS1 mice; the first deposits were identified at 6 months, mainly within frontal and entorhinal cortices and hippocampus. These deposits were also associated with reactive astrocytes and dystrophic neurites. Diffuse deposits in

striatum, thalamus and brainstem were defined in 12 month old APP/PS1 mice.

Progression of deposition density and number of diffuse plaques occurred at 15 months; however no deposits were found in granular and pyramidal cell layers of the hippocampus or the corpus callosum. At this late age, GFAP staining for reactive astrocytes was greatly increased throughout the brain, mainly concentrated in the striatum and cerebral cortex, compared to their first detection from 6 month old mice (Gordon et al., 2002).

Although Borchelt et al. (1997) also detected substantial A β deposition within cortex and hippocampus in 12 month old APP/PS1 mice, no deposition was found in any other region in contrast to Gordon et al. (2002) that found plaques in striatum, thalamus and brainstem. Borchelt et al. (1997) also reported a progression of deposition from 9 to 12 months with increased number of plaques within hippocampus and occipital and frontal cortices. In conjunction with the previous studies, many of the deposits were associated with reactive astrocytes (Borchelt et al., 1997). Overall, the APP/PS1 transgenic model develops modest A β deposition associated with reactive astrocytes around 3-6 months of age with a progression in density and number within cortex and hippocampus thereafter.

Numerous behavioral studies have been performed similar to the previously discussed models to characterize the development of cognitive impairment. At 3 months of age, APP/PS1 mice were found to exhibit no deficits in sensorimotor tasks, but did become impaired in Y-maze alternation and exhibit increased activity in Y-maze entries by 6 to 9 months of compared to age-matched non-transgenic mice (Holcomb et al., 1999). However, even at 9 months there were no sensorimotor deficits or memory

deficits revealed by visible platform or water maze acquisition or retention trials (Holcomb et al., 1999).

By contrast Arendash et al. (2001b) found no difference of APP/PS1 mice from non-transgenics at 5-7 months in Y-maze alternation; however, they did have increased activity as measured by Y-maze entries. The same study also found impairment in a balance beam task at this age with no impairments in any additional tasks including sensorimotor and learning or memory tasks (Arendash et al., 2001b). At 15-17 months of age, double transgenic mice exhibited increased activity in open field and Y-maze entries versus non-transgenics with additional impairments in balance beam, string agility, Morris water maze acquisition and radial arm water maze (RAWM) working memory (Arendash et al., 2001b). Within this study correlation analyses revealed a significant positive relation between working memory errors in the radial arm water maze task and Congo red staining in the frontal cortex (Gordon et al., 2001). There was also a significant negative correlation between total A β load in the frontal cortex and hippocampus and radial arm water maze acquisition error reduction between trial 1 and trial 4 (Gordon et al., 2001).

Of the three AD transgenic models presented, the APP/PS1 mouse has certain advantages, including considerably earlier A β deposition and a comprehensive behavioral evaluation at multiple time points. It is, therefore, ideally suited for studies of this dissertation, which involve the testing of vaccination and dietary therapeutics against behavioral impairment and AD neuropathology provided by that model.

IX. AD Vaccination

Current treatment options for AD include cholinesterase inhibitors, NSAIDs, statins and a variety of antioxidants as previously reviewed. However, these therapies are largely aimed at the symptomatic basis of the disease instead of the underlying pathology involved. Utilization of immunotherapy as a possible treatment of AD involves inducing an immune response to fight misfolded proteins or aggregates of proteins that accumulate in the pathogenesis of AD. Therefore, immunotherapeutic approaches toward AD hold valid opportunities to treat this neurodegenerative disease through a humoral or cellular immune-mediated response.

In Vitro Studies.

In 1996, Solomon et al. first experimented with monoclonal antibodies (mAbs) to test their effectiveness against A β peptide aggregation, fibrillization and toxicity in vitro. They found that mAbs against A β_{1-28} were effective at converting fibrillar A β to an amorphous state. In 1997 Solomon et al. then showed that the mAb 6C6 against A β_{1-16} significantly solubilized fibrillar A β in comparison to control cells. Starting in 1998 Frenkel et al. began to determine the specific epitope for antibody binding which was responsible for completely inhibiting A β fibril formation. They determined that the EFRH epitope corresponding to A β_{3-6} was most effective at solubilizing fibrillar A β . These results support the studies previously performed by Solomon et al. (1996, 1997), who determined that mAbs against A β_{1-28} or A β_{1-16} were more successful than mAbs against A β_{8-17} or A β_{13-28} for disaggregating A β fibrils. Following these studies were A β

immunotherapy experiments in multiple transgenic models to determine the efficacy of previously determined *in vitro* results in an *in vivo* environment.

Active A β Immunotherapy.

Schenk et al. (1999) first explored active A β immunization by administering the A β_{1-42} peptide to 6 week old PDAPP mice once monthly until 13 months of age. The vaccination sequence resulted in almost complete prevention of A β deposition. Immunization of 11 month PDAPP mice until 15 and 18 months resulted in arrest of total A β deposition within the cortex to the level present at 11 months. Immunization resulted in the generation of blood anti-A β antibodies and activation of microglia found near plaques; therefore one possible mechanism for A β clearance, before or after plaque formation, could be due to anti-A β antibodies triggering microglial cells to clear A β through an Fc receptor-mediated phagocytosis. Schenk et al. (1999) concluded that active immunization could be used as both a prevention and therapy to block or retard the pathological development within an AD-like pathology. Disappointingly, there were no behavioral measures performed on these mice to determine if the improved pathology could also retard behavioral impairments. In 2001, Dickey et al. vaccinated 5-month old APP+PS1 mice with the A β_{1-42} peptide monthly for 7 months. They found that more than three vaccinations are necessary to induce a 50% maximal antibody titer that progressively increased after the 6th booster vaccination. A competition binding inhibition assay was used to determine binding efficacy and specificity of the sera from the immunized mice. A β_{1-16} was the only truncated A β peptide from the sera that

inhibited binding of antibodies to A β ₁₋₄₂. Also, the A β ₁₋₄₂ was more effective than A β ₁₋₄₀, indicating the importance of the 2 amino acid difference between the peptides in antibody binding. Because A β ₁₋₄₂ forms fibrils more readily than A β ₁₋₄₀, this conformational change could significantly contribute to the preferential recognition by the immune system. Lastly, T-cell proliferation was increased in spleen cells of APP+PS1 vaccination mice (Dickey et al., 2001).

Recently, Koller et al. (2004) vaccinated APP mice with A β ₁₋₄₂ cross-linked with an Hsp70 homolog of *Escherichia coli*, DnaK, beginning at 6 weeks of age (prior to onset of plaque deposition), with 3 inoculations ending at 12 weeks of age. In opposition to Dickey et al. (2001), measurable antibody titers against A β ₄₂ were detected after only one inoculation with the *DnaK*-A β ₄₂ vaccination. At 6-9 months, brain levels of A β were determined for human and murine A β in brain homogenate. The *DnaK*-A β ₄₂ immunized mice showed a surprising increase of extractable A β indicating an increase of A β aggregates as a result of the vaccination. This change occurred without any significant plaque deposition. By 12-14 months of age, both *DnaK*-A β ₄₂ immunized and control APP mice showed low levels of plaque burden in the neocortex with no difference between the two groups. Also at this age, the extractable total A β and A β ₄₂ were increased in the brains of the *DnaK*-A β ₄₂ immunized mice compared to the non-immunized APP mice. Both immunized and non-immunized APP transgenics exhibited A β deposits in the walls of cerebral blood vessels. There was a trend toward a slightly higher A β deposition in the brains of immunized mice, as evidenced by their having A β deposits within smaller cerebral blood vessels in addition to A β deposits in large cerebral

blood vessels. The authors propose a possible link between the increased A β aggregates resulting from vaccination and the non-significant increase in A β within small cerebral vessels. The authors propose that this link may be a direct result of the vaccination, however there is no significant evidence to support this association.

Monsonogo et al. (2001) immunized APP Tg2576 transgenic mice with A β_{1-40} at 5 weeks of age, with blood samples collected ten days later for antibody and T-cell analysis. Immunized APP mice had lower levels of anti-A β antibodies compared to immunized wild-type littermates; the APP control vaccinated mice did not produce any detectable levels of anti-A β antibodies. The A β_{1-40} APP immunized mice also had decreased T-cell proliferation, INF- γ , and IL-2 secretion compared to non-transgenic littermates. In opposition to Dickey et al. (2001), Monsonogo et al. (2001) did not show an increase in T-cell proliferation. The difference could largely be due to the number of inoculations given, type of vaccination, and/or the difference in transgenicity used in the animal models. The sera from the APP immunized mice were used to positively stain neuritic plaques in brains of mature APP mice; sera from non-immunized mice did not positively stain any neuritic plaques. The authors suggest that the APP immunized mice exhibit a hyporesponsive immune response to the A β_{1-40} vaccination, as evident by reduced T-cell and anti-A β antibody production. They relate this impaired immune response of the APP mice to humans that have chronic elevations of brain and peripheral A β , resulting in their also exhibiting an impaired immune response to A β vaccinations.

Aside from the previous studies that used direct injections of the A β_{1-42} peptide, Weiner et al. (2000) performed nasal administration of the less amyloidogenic A β_{1-40}

peptide to 4.5 month old PDAPP mice once weekly for 7 months. Mucosal administration has the ability to induce an anti-inflammatory immune response which is antigen-specific within lymphoid tissue. This response then acted systemically to significantly reduce A β deposition within cortical and hippocampal tissues. This occurred in conjunction with a decrease in microglia and astrocyte activation within these areas (Weiner et al., 2000). More recently, Lemere et al. (2003) showed that 5 week old APP+PS1 mice immunized intranasally with a cocktail of A β peptides (3 parts A β ₁₋₄₀, 1 part A β ₁₋₄₂) twice weekly for 8 weeks exhibited a 75% reduction in cerebral A β plaque number and 58% decrease in neurochemical levels of A β ₁₋₄₂ in the brain. There was also a large increase in serum A β antibody levels compared to untreated control transgenics. The authors concluded that because most of the serum A β was bound to antibodies, that the antibodies attached to serum A β to aid in clearance.

McLaurin et al. (2002) vaccinated TgCRND8 mice with protofibrillar aggregates of A β ₁₋₄₂ or control vaccinations. This resulted in recognition of A β ₄₂ monomers, tetramers, hexamers and large oligomers by A β ₄₂-immune sera. The A β ₄₂-immune sera did not activate the Th1 helper cell pro-inflammatory cascade, but instead the Th2 helper cell response was activated, which promotes B cells to make anti-A β antibodies. Specifically, they found that A β ₄₋₁₀ peptide was highly recognized by the anti-A β ₄₂ IgGs. A β fibril formation and toxicity were inhibited by anti-A β ₄₋₁₀ without activation of surrounding microglia. Overall, McLaurin et al. (2002) concluded that using small-molecules could decrease some of the detrimental problems seen in immunotherapy such as a pro-inflammatory response. This study had similar results compared with previous

work by Frenkel et al. (1998), who first reported that the epitope of A β ₁₋₄₂ is the A β ₄₋₁₀ segment; Frenkel et al. proposed the segment specifically corresponded to A β ₃₋₆.

Additional active immunization animal studies have been done to prevent against AD progression. These studies administer A β active immunization prior to A β deposition in transgenic models and behaviorally assess changes that could occur in conjunction with A β pathology. Vaccination of 6 week old TgCRND8 mice with A β ₄₂ for 3½ months resulted in a 50% decrease in the number and size of A β -positive dense core plaques within the hippocampus and cortex (Janus et al., 2000). At various age points from 11 to 23 weeks, Morris water maze testing was done; improved performance of the A β ₄₂ vaccination group in comparison to control immunized mice was observed at several, but not all, test points (Janus et al., 2000). Morgan et al. (2000) began administration of A β ₁₋₄₂ peptide to APP+PS1 transgenic (Tg+) mice at 5-7 months of age — prior to A β deposition and behavioral deficits. The inoculations were given monthly until 15.5 months of age. High antibody titers were found in immunized Tg+ and non-transgenic (Tg-) mice; control vaccinated and untreated mice did not produce any detectable antibodies to A β ₄₂, indicating that transgenic mice do not spontaneously produce anti-A β antibodies. A modest, non-significant, decrease in A β burden in the frontal cortex was produced in the brains of Tg+ immunized mice; however, there was a significant reduction in Congo red-positive compact plaques within the frontal cortex of Tg+ immunized mice. Trends toward reduced A β burden and Congo red staining were found in the hippocampus of vaccinated mice. All mice were behaviorally pretested before vaccination began, ~5-7 months of age, and similarly behaviorally tested at ~16

months of age after 8 months of A β inoculations. At the 16 month test point, vaccinated Tg+ mice performed similar to Tg- mice in radial arm water maze working memory, and significantly better than control Tg+ mice (Morgan et al., 2000). Despite 8 months of vaccinations, Tg+ mice remained impaired in balance beam compared to Tg- mice (Arendash et al., 2001a). In order to relate A β deposition to behavioral performance, correlation analyses were performed. These analyses showed significant correlations between A β_{1-40} deposition in hippocampal region CA1 and radial arm water maze working memory impairment for all Tg+ mice grouped together. Additionally, A β_{1-40} deposition and Congo red staining in frontal cortex correlated with radial arm water maze working memory impairment for both Tg+ groups combined. The authors suggest that the behavioral protection offered by an A β vaccine selectively and primarily preserves hippocampal-associated working memory function (Arendash et al., 2001a). Additionally, the authors propose that the significant prevention of memory deficits exhibited in the radial arm water maze task, coupled to the slightly reduced but still substantial A β load, could be due to the ability of A β antibodies to neutralize soluble, non-deposited A β (Morgan et al., 2000). This type of A β has been implicated in memory loss due to synapse loss in dentate gyrus. A second proposed theory to account for the observed results is through a clearance of deposited A β by activated microglia; however the lack of significant difference in A β deposition between the vaccinated and control transgenic groups does not support this theory (Morgan et al., 2000).

Recently, Sigurdsson et al. (2004) immunized 6 to 8-month old Tg2576 APP mice with monthly inoculations of nontoxic A β_{1-30} or K6A β_{1-30} until 19 to 21 months of

age. K6A β 1-30 binds specifically to residues 1-11 and 22-28 of A β ₄₂ without adopting a β -sheet conformation and is therefore less toxic. Immunization with A β 1-30 produced no difference in amyloid plaque burden or soluble A β levels. Likewise, vaccination with K6A β 1-30 to the APP mice did not produce a significant change in overall amyloid burden; however, there were reductions in small and medium-sized plaques in the vaccinated group compared to control-immunized APP mice. There was also no change in soluble A β levels between the K6A β 1-30 mice and the control group. In addition to the pathologic markers, Sigurdsson et al. (2004) showed that APP mice vaccinated with K6A β 1-30 had improved cognitive performance, as shown by a decrease in errors in the radial arm maze task compared to control vaccinated APP mice. Both the K6A β 1-30 vaccinated and control wild-type mice exhibited a learning effect, as shown by a decrease in errors from day 1 to day 9; the control vaccinated APP mice did not show any learning effect. Overall, because the K6A β 1-30 vaccinated mice showed an improvement in learning with minimal changes in amyloid burden, the authors suggest that this type of immunization clears A β oligomers which are linked to behavioral performance.

In comparison to vaccination studies for prevention against AD, few studies have focused on the use of immunization as a treatment option for AD. Wilcock et al. (2001) immunized 3 groups of APP+PS1 mice with the A β ₁₋₄₂ peptide beginning at 7.5, 13 and 14.5 months of age for 9, 3 and 5 months, respectively. Overall, all groups exhibited decreases in Congo Red staining in hippocampus, but not in frontal cortex. In another study, Sigurdsson et al. (2001) vaccinated 11-13 month old Tg2576 mice with a nonamyloidogenic, non-toxic A β homologous peptide for 7 months until the age of 18-20

months. This resulted in decreased amyloid burden in cortex and hippocampus as well as decreases in Congo Red staining and soluble A β ₄₂ deposition in comparison to control vaccinated mice. In opposition to Wilcock et al. (2001), a decrease in Congo red staining was observed in frontal cortex. The discrepancy between Wilcock and Sigurdsson is most likely due to the difference in vaccination protocol used between the two groups, non-toxic A β homologous peptide versus A β ₁₋₄₂ peptide.

In 2003, Frenkel et al. injected the EFRH epitope, corresponding to A β ₃₋₆, into 16-month old PDAPP mice monthly for 4.5 months. In agreement with Dickey et al. (2001), antibody titers reached high levels after 6 injections. They demonstrated that monoclonal antibodies that disaggregate A β also bind to the EFRH-phage; conversely, monoclonal antibodies that do not disaggregate A β , do not bind to the EFRH-phage. Incubation of prepared A β fibrils with anti-EFRH sera resulted in disaggregation of the fibrils into an amorphous state. Likewise, plaques from the hippocampus of PDAPP mice and AD human subjects were positively stained with the anti-EFRH anti-sera. Analysis of 21-month old PDAPP vaccinated mice showed a significant reduction in amyloid burden compared to the control-vaccinated PDAPP mice. The authors suggest that the EFRH-phage induced an auto-immune response by PDAPP mice to produce anti-EFRH that binds and disaggregates A β fibrils to reduce the number of A β plaques in the brain (Frenkel et al., 2003).

Austin et al. (2003) performed biweekly injections of A β ₁₋₄₂ for 6 weeks in cognitively impaired 16 month old APP+PS1 mice. Mice were pre-tested in radial arm water maze and platform recognition tasks prior to the first vaccination. All Tg⁺ mice

exhibited impaired working memory retention within both tasks. Within the platform recognition task, overall both groups of Tg+ mice had increased latency compared to Tg- mice, but by the end of the task (day 4) they exhibited similar performance to Tg- mice. Following the vaccination period, aged transgenic mice exhibited working and reference memory impairment in the radial arm water maze task that was not corrected with A β immunization. However, both groups of Tg+ still showed similar performance to Tg- mice in platform recognition by having decreased latencies by day 4 in this task. By 20 months of age after the final behavioral assessment, anti-A β antibody levels were detected in all Tg+ mice that were vaccinated; in opposition, no antibody levels were detected in control vaccinated mice. The authors concluded that a longer vaccination immunotherapy might provide a better cognitive advantage to aged APP+PS1 mice.

Passive A β Immunotherapy.

Passive immunization studies involving injection of antibodies against A β ₁₋₄₂ have been performed in A β -depositing transgenic mouse models. Pfeifer et al. (2002) suggest that the binding of an antibody to soluble A β has the potential to cause a local inflammatory reaction and can destabilize weakened vessel walls. Transgenic APP23 mice (which develop amyloid angiopathy) were immunized with a monoclonal antibody against A β that recognized A β ₃₋₆. This resulted in a significant increase in hemorrhage severity mainly within amyloid-rich vessels. The authors suggest that the weakening of the vessels could be due to an A β -induced increase in vascular permeability, resulting in the loss of smooth muscle cells. In addition, they suggest that an increase of antibody

binding to soluble A β in the blood could lead to increased risk of cerebral hemorrhage.

However, no other passive immunization studies use mouse models that develop cerebral amyloid angiopathy and therefore do not show an increased risk for cerebral hemorrhage to support these findings. Also, the immunization resulted in a 23% decrease of A β load in neocortex, mainly due to a reduction of diffuse deposits (Pfeifer et al., 2002).

Bard et al. (2000) however, showed that PDAPP mice immunized weekly with monoclonal antibodies, 10D5 or 21F12, beginning at 8 to 10 months of age for 6 months had more than an 80% decrease in plaque burden within frontal cortex with the 10D5 antibody. The 10D5 monoclonal antibody is specific to A β residues 3-6, whereas 21F12 is specific to A β ₄₂ residues 34-42. The same group also immunized 11.5 to 12 months old PDAPP mice with monoclonal antibodies, 3D6 and 16C11 for 6 months. A significant decrease in frontal cortex plaque burden was seen only with 3D6 and in the absence of T-cell proliferation within splenocytes. 3D6 is specific to A β residues 1-5, whereas 16C11 is specific to A β residues 33-42. The authors concluded that this type of passive immunization against A β is sufficient to induce decreases in amyloid deposition without T-cell mediated cellular immunity. In agreement with Schenk et al. (1999), these authors suggested that the mechanism for A β clearance involves antibodies entering the CNS and binding to Fc receptors on microglial cells to trigger phagocytosis of deposited A β peptide (Bard et al., 2000).

DeMattos et al. (2001) vaccinated 4-month old PDAPP mice for 5 months with the m266 monoclonal antibody, which is specific to A β ₁₃₋₂₈. M266 vaccinated mice had marked reductions in A β deposition in the cortex and dorsal hippocampus compared to

control vaccinated PDAPP mice. Despite the reduction of A β deposition, m266 did not bind to A β deposits within the brains of PDAPP mice. In addition, A β levels in the plasma were increased 1000-fold in vaccinated transgenics compared to control mice. Since, m266 does not cross the blood brain barrier, the authors suggested that m266 acted as an A β sink to bind and sequester A β from the CNS to significantly increase plasma A β to aid in clearance. Specifically, the authors suggested that m266 altered the plasma and brain A β equilibrium to favor A β in the plasma and therefore reduce deposition in the brain (DeMattos et al., 2001).

An additional passive immunization treatment study involved a single ICV injection of a monoclonal antibody AMY33 (specific to A β_{1-28}), into the third ventricle of 10-month old Tg2576 APP mice (Chauhan & Siegel, 2003). The authors claim that AMY33 immunization reduced the size of compact plaques, at one-month after the injection, compared to control vaccinated APP mice; however, the figures do not support this statement and no quantification of plaques was performed. There seems to be no difference in staining between the treatment and control groups. The study also claimed that AMY33 immunization decreased the number of immunoreactive microglia surrounding compact cerebral plaques. Overall, AMY33 does not seem to be effective at reducing AD-related pathology such as A β burden or microglial response.

A similar short-term passive vaccination study topically applied the anti-A β antibody 10D5 by craniotomy to the cortex of 19-23 month old PDAPP mice (Lombardo et al., 2003). The antibodies were only effective to the immediate cortical area where they are applied; remote areas from the application site were not affected by the antibody. Amyloid burden was decreased significantly only in the immediate area of application at

4 and 32 days after topical treatment with 10D5. Also in the immediate area from 4 to 32 days post-treatment, neurites from 10D5 immunized PDAPP mice appeared normal similar in fact to those in non-vaccinated Tg- mice; neurites in a remote area of PDAPP mouse brains (not exposed to 10D5) had a greater curvature ratio. The authors concluded that the curvature neurite abnormalities exhibited in the remote area of PDAPP brains are linked to A β deposition and that these neurites undergo rapid restoration to a normal morphology following A β plaque clearance (Lombardo et al., 2003).

Kotilinek et al. (2002) found that intraperitoneal administration of BAM-10 (a monoclonal antibody that recognizes A β residues 1-12) for one week resulted in no change in neurochemical A β_{40} or A β_{42} levels within brains of 9-11 month old Tg2576 mice versus Tg+ controls. However, the BAM-10 group did perform significantly better in Morris water maze acquisition and probe trial retention compared to a pretreatment performance within the same group. The authors propose that toxic A β assemblies or oligomers are responsible for cognitive impairment seen in Tg+ mice; therefore, neutralization of these A β species could lead to cognitive improvement. This is the proposed mechanism of action of BAM-10 since resulting improvement in memory/learning of Tg+ mice occurred without alterations in brain A β levels.

In contrast to the prevention study performed by DeMattos et al. (2001), Dodart et al. (2002) found that a 6 week treatment with the antibody m266 in 24 month old PDAPP mice resulted in no change in A β burdens in hippocampus or cortex, but the vaccinated mice given high doses of m266 did perform significantly better in an object recognition task than Tg+ mice given PBS injections. Since m266 does not cross the blood-brain

barrier, the mechanism is thought to be through the peripheral clearance or sequestration of soluble A β in order to efflux A β from the brain. This theory stems from the detection of an A β /m266 complex in the plasma and CSF at high doses of m266. The authors suggested that the high doses are necessary for m266 to enter the central compartment and draw soluble A β from the brain into the plasma (Dodart et al., 2002).

More recently, Wilcock et al. (2004) vaccinated 19-month old Tg2576 APP mice weekly with the anti-A β antibody 2286 (specific to A β 28-40) for 1, 2, or 3 months. Serum A β antibodies were detected at high levels after 1 month of vaccination and associated with high circulating serum A β levels. However, by 2 and 3 months of vaccination, serum A β decreased, but remained elevated compared to control vaccinated APP mice. Both compact and diffuse A β deposition were reduced in the cortex by ~60% and hippocampus by ~55% of APP mice vaccinated for 2 and 3 months compared to control vaccinated APP mice. Also, transient microglial activation was seen in vaccinated APP mice at 2 months of treatment compared to control APP mice. By 3 months of treatment, microglial activation returned back to levels found at 1 month of treatment. At 22 months of age, a behavioral assessment was done to evaluate the effects of the vaccination. All groups were tested in the Y-maze task, which indicates general memory function and activity. APP vaccinated mice had improved performance shown by a higher percent alternation compared to APP control vaccinated mice. Also, the APP control mice, in addition to APP mice vaccinated for 1 month, had significantly increased arm entries versus non-transgenic mice. However, following 2 or 3 months of vaccination treatment, APP mice exhibited similar activity levels to that of the non-

transgenic mice. The authors suggested that anti-A β antibodies are able to enter the brain and activate Fc receptors on microglial cells to stimulate phagocytosis, as first described by Schenk et al. (1999).

A β Immunotherapy in Humans.

Following the evidence that both active and passive immunization of mouse models decrease A β deposits within brain regions and reduce cognitive deficits, human trials began with immunization of A β ₁₋₄₂ (AN-1792) by Elan and Wyeth-Ayerst Laboratories. After a few months during Phase II trials, about 5% of the participants receiving the vaccine developed severe inflammation in the spinal cord and brain, so the trials were halted. However in a Zurich cohort there was no significant correlation between the production of antibodies and the incidence of aseptic meningoencephalitis (Hock et al., 2003). This trial included 30 participants with mild to moderate AD; 24 patients received the vaccination of AN1792 and developed antibodies. After all the trials were halted, antibody production and reactivity, due to the vaccination, were analyzed. They discovered that human sera produced antibodies that reacted with β -amyloid plaques and vascular amyloid on brain sections of APP+PS1 transgenic mice and β -amyloid from human brains (Hock et al., 2002). Following a one-year follow-up, 19 of 28 patients that developed antibodies showed a stabilization of cognitive performance, as measured by the MMSE, compared to subjects that did not produce antibodies and exhibited cognitive decline. The patients that developed antibodies also had preserved hippocampal function as shown through the Visual Paired Associates Test versus patients that did not develop antibodies. Lastly, due to unchanged CSF and

plasma levels of A β ₄₀ and A β ₄₂, the antibodies produced from the vaccine did not sequester serum A β in order to alter cognition (Hock et al., 2003). Despite the above beneficial results from human A β ₁₋₄₂ vaccination, the serious inflammatory complications from the vaccine do not allow for additional human trials with this approach. Passive immunization or vaccinations with modified forms of A β have been recommended to circumvent some of the problems associated with active immunization, such as stroke, encephalitis and sterile meningitis (McGeer & McGeer, 2002). Proposed mechanisms that could account for the unexpected inflammatory reactions seen in a few of the patients in the clinical trials include: 1) autoimmune disorder due to antibody reactivity with host proteins and 2) autodestruction due to the host cells being damaged by the membrane attack complex (MAC). Under normal conditions antibodies are generated against foreign material; however spontaneous generation against host proteins can lead to disorders known as autoimmune diseases. Autodestruction occurs when MAC, which normally protects host cells by attacking bacterial and viral pathogens, is over activated as occurs during AD; this results in more complement activation and destruction of neurons leading to an increase of damage to host cells (McGeer & McGeer, 2002).

IX. Omega-3 Fatty Acids

General Background.

Omega-3 fatty acids are found mainly enriched within fish and fish oil supplements. The specific fatty acids at high concentrations within these foods include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Omega-3 fatty acids can

also be found in hemp oil, flax oil and green leaves (Haag, 2003). Retrospective studies indicate that a high intake of n-3 fatty acids is associated with a reduced risk of dementia, specifically AD, prevention of cardiac arrhythmias, through reduced thrombosis, decreased inflammation including arthritis and decreased weight loss to reduce the risk of death associated with cancer and AD (Kalmijn et al., 1997a; Kalmijn et al., 1997b; Horrocks and Yeo, 1999; Knittweis, 1999). Omega-6 fatty acids are found mainly within meats and vegetable oils such as sunflower oil, evening primrose oil, corn oil and safflower oil (Haag, 2003). Arachidonic acid, an omega-6 fatty acid, can specifically be found in meats, eggs, shrimp and prawns (Das, 2003).

Essential fatty acids (EFAs) include linoleic acid (LA) and α -linolenic acid (ALA). These fatty acids can not be produced *de novo* and must be provided by dietary intake. All of the polyunsaturated fatty acids (PUFAs) are synthesized from these two EFAs; LA gives rise to all of the omega-6 fatty acids while ALA synthesis results in all of the omega-3 fatty acids. Fatty acids are named to identify the structure of the molecule so that DHA is denoted as 22:6n-3. The first number, 22, refers to the number of carbon atoms in the hydrocarbon backbone, the 6 represents the number of double bonds and the 3 gives the position of the last double bond. N is equal to the number of total carbon atoms so that n-3 represents the number of carbon atoms from the last double bond to the terminal methyl group.

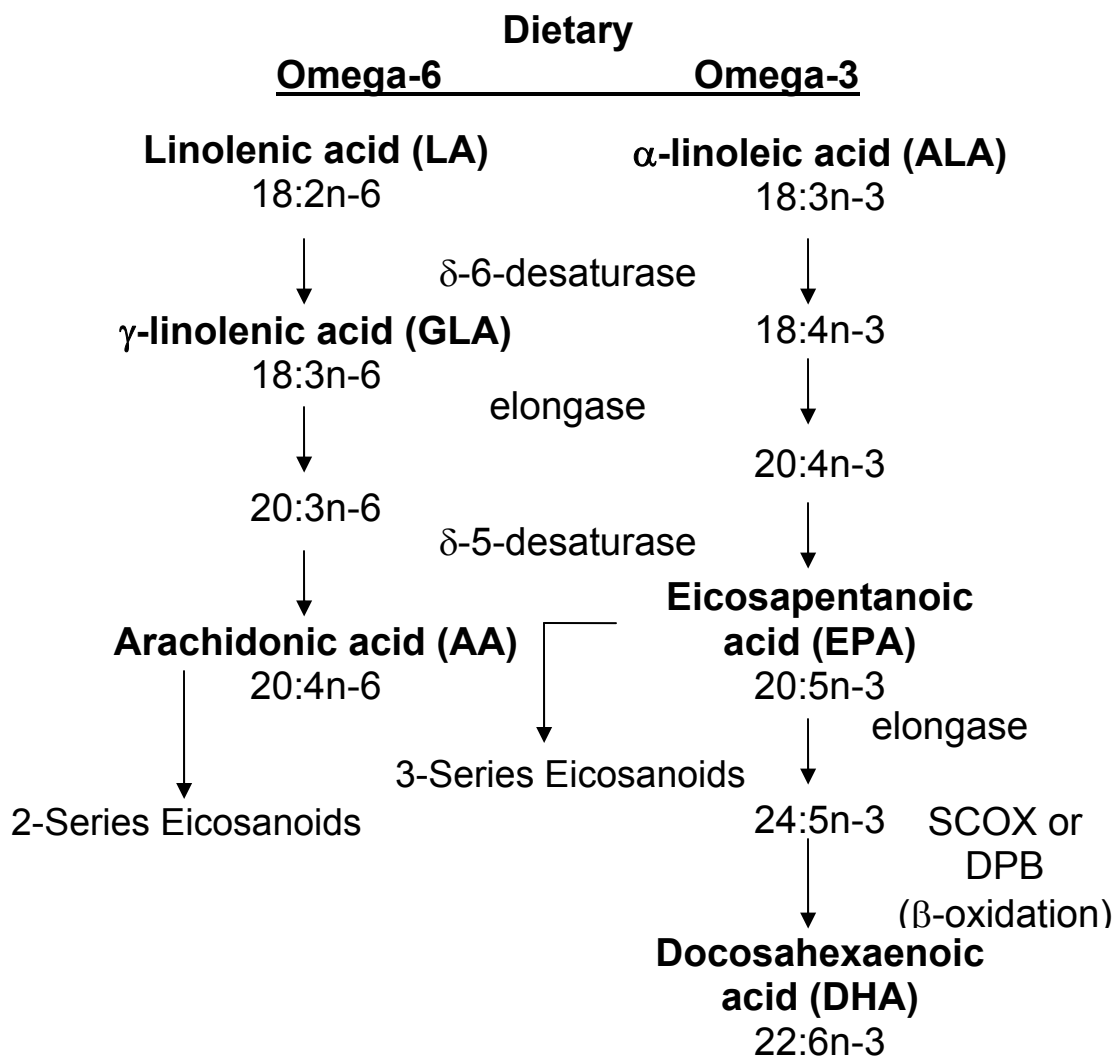
As indicated from animal studies, the primary supplier of brain DHA levels is from the plasma. Plasma DHA is either obtained from dietary intake or from precursors synthesized into DHA by the liver. Additional studies indicate that DHA can be synthesized directly from the brain and its concentration can be regulated by n-3

precursors or the availability of preformed DHA. Specifically, DHA synthesis occurs within astrocytes, which provide DHA in high concentrations to neurons and blood-brain barrier endothelium cells (Williard, et al., 2001).

Because mammals do not possess the enzyme omega-3 desaturase they can not convert omega-6 fatty acids into omega-3 fatty acids. The synthesis of fatty acids within the liver consists of desaturation and elongation reactions within the endoplasmic reticulum and peroxisomes as illustrated below in Figure 2. Initially, ALA receives an additional double bond to change from an 18:3n-3 fat into an 18:4n-3 fatty acid by δ -6-desaturase. Elongation of the carbon chain occurs to produce 20:4n-3 which is then altered by δ -5 desaturase to yield EPA (20:5n-3). EPA is then elongated by two separate reactions to produce 24:5n-3. Δ -6 desaturase then adds another double bond to produce 24:6n-3, which undergoes β -oxidation to yield DHA (22:6n-3) (Ferdinandusse, 2001; Haag, 2003). Unfortunately only 5% of the dietary ALA is ultimately converted into DHA (Sarsilmaz et al., 2003). Initially it was proposed that all of the fatty acid conversion to DHA were performed within the endoplasmic reticulum (ER), however recent research has proven that the final β -oxidation step to yield DHA occurs within the peroxisomes. The potential enzymes responsible for this conversion have been identified as either a straight-chain acyl-CoA oxidase (SCOX) or a α -bifunctional protein (DPB) (Ferdinandusse, 2001). Omega-6 fatty acid conversion from LA (18:2n-6) undergoes the same path using the same enzymes to ultimately yield 20:4n-6 (arachidonic acid, AA).

Additional products are also produced at several stages in the pathway by both omega-3 and omega-6 fatty acids. Di-homo-linoleic (DGLA) acid, an n-6, can give rise to precursors for 1-series eicosanoids. Eicosanoids encompass prostaglandins and

thromboxanes. Arachidonic acid (AA), also an n-6, can form precursors to 2-series eicosanoids, while EPA, an n-3 fatty acid, can produce precursors for 3-series eicosanoids (Fig. 2). The 2-class is pro-inflammatory whereas the 3-class of eicosanoids is anti-inflammatory in function (Das, 2003).



Dietary Manipulation of n-3 and n-6 Fatty Acids.

Most of the impacts from fatty acids occur following their incorporation into cellular membranes. DHA has the potential to influence membrane fluidity, hormone production, enzyme activities and the formation of lipid peroxidation products (Stillwell & Wassall, 2003). While affinity for DHA incorporation into membranes is highest in retinal cells (Alessandri, 2003), it is also present in synaptosomes and sperm cell membranes (Stillwell & Wassall, 2003), whereas the hippocampus and cerebral cortex show the lowest affinity for DHA membrane incorporation (Alessandri, 2003). Heart, skeletal muscle, liver and kidney membranes also contain levels of DHA within their phospholipids (Turner et al, 2003a). Particular phospholipids (PL) classes incorporate DHA at distinct rates and affinities. Stillwell and Wassall (2003) determine that within plasma membrane and mitochondria, DHA first integrates into phosphatidylethanolamine (PE) followed by incorporation into phosphatidylcholine (PC) and the remainder of the PL classes. Within these two PL classes, cerebral cortical cell membranes from hamsters fed an omega-3 deficient diet contained significant decreases in DHA and docosapentaenoic acid (DPA) levels as well as total n-3 fatty acids. Supplementation of these cells with DHA resulted in increases of DHA and EPA in a dose-dependent manner in addition to a decrease of AA and total n-6 fatty acids (Champeil-Potokar et al., 2004). In another study, synaptic plasma membranes from ApoE-deficient mice contained an increased amount of DHA within PC and phosphatidylserine (PS) classes possibly due to an overcompensation of DHA transport due to the decreased transport of other PUFAs from the ApoE deficiency (Igbovboa, 2002).

Animal studies show that aged rat (21-24 month) hippocampus (Favreliere et al., 2003) and whole brains (Barcelo-Coblijn et al., 2003) have significantly less DHA in PE, phosphatidylserine (PS) and plasmalogen phospholipids classes versus young rat (2 month) brains. Within some phospholipid classes, such as phosphatidylinositol (PI), sphingomyelin and PS, the levels of AA, C16 (palmitic acids), total n-6 and n-3 fatty acids have also been shown to be lower in aged rat (27 month) whole brains in comparison to adult rat (7 month) brains; there were no differences shown specifically in hippocampus (Ulmann et al., 2001). Old rats supplemented with fish oil for one month had restored levels of DHA within ethanolamine phosphoglycerides of whole brains compared to the levels in young control-fed rats (Barcelo-Coblijn et al., 2003). In addition, 3 months of DHA supplementation to adult rats (18 month) resulted in significant increases of AA, DHA and total n-6 PUFAs in hippocampal phospholipid classes, PE and PmE, in comparison to unsupplemented control fed rats (Favreliere et al., 2003). Young mice have even benefited from supplementation with fish oil. Puskas et al., (2004) supplemented 4½ month old mice for 2 months with cholesterol alone, cholesterol plus fish oil, or standard chow. Mice that were supplemented with only cholesterol had a reduced amount of DHA in whole brain, whereas mice fed both cholesterol plus fish oil had increased DHA in whole brain and retina as compared to mice fed the standard chow. In addition to measuring fatty acid levels in the brain and retina, this study also analyzed gene expression, specifically peroxisome proliferators-activated receptors (PPARs), sterol-regulatory element binding proteins (SREBPs), fatty acid binding proteins (FABPs) and inflammatory proteins, all of which will be discussed in the following sections.

Many animal studies focus on young animals and involve n-3 deficiency or supplementation for several generations. Two similar studies by Ikemoto et al. illustrate that rats fed an n-3 deficient (safflower oil as main fat source) diet for two generations supplemented with either DHA (2001) or EPA (2000) exhibit a restored level of DHA within the whole brain compared to control mice fed an n-3 sufficient diet. The n-3 deficiency led to increased levels of n-6 PUFAs, AA and 22:5n-6 within whole brain compared to supplementation with either DHA or EPA (Ikemoto et al., 2000; Ikemoto et al., 2001). Gamoh et al. (2001) demonstrated that rats fed a fish oil-deficient diet for 2 generations and subsequently supplemented with DHA showed a significant increase in DHA content and DHA/AA ratio within the cerebral cortex, with no difference between fatty acid levels within the hippocampus. Similarly, Moriguchi et al. (2001) fed rats either an n-3 adequate or n-3 deficient diet for 2 generations. The F2 generation rats on the n-3 deficient diet were weaned onto the n-3 adequate diet at 7 weeks of age and then groups were sacrificed immediately and every 2 weeks thereafter for 8 weeks. Specifically, this study measured the recovery rate of serum, retina, liver and brain of fatty acid levels after 2 generations on an n-3 deficient diet, as compared to being raised on an n-3 adequate diet. For the serum, recovery of n-3 fatty acids to levels seen in the n-3 adequate rats were accomplished after 2 weeks of switching diets. The liver, was the most rapidly recovering of all the tissue measured in that liver n-3 levels were comparable to those of the n-3 adequate group after only 1 week. In contrast, the brain had a slower recovery rate, such that even after 8 weeks of being fed an n-3 adequate diet, levels of DHA were still not comparable to the mice originally fed the n-3 adequate diet. With a faster recovery than the brain, the retina had full recovery of DHA in the rats

that had been on the n-3 adequate diet for 8 weeks. The authors analyzed the recovery rate for each of the tissues in order to normalize the data. They found that the serum actually had the slowest recovery rate per tissue weight, which was most likely a reflection of the low content of DHA in the serum compared to the other tissues. The brain and retina had higher recovery rates than the plasma with respect to tissue weight. Because of their higher recovery rates yet longer recovery time within the brain and retina; this indicates an inability in uptake or delivery of fatty acids to rapidly replenish these areas of the nervous system (Moriguchi et al., 2001).

Even without generations of n-3 deficiency, many animal studies show significant changes in fatty acid composition within plasma or brain lipids following an n-3 deficient diet. In general, rodents fed an n-3 deficient diet exhibit decreases in total n-3 PUFAs including DHA and EPA as well as increases in total n-6 PUFAs such as AA; diets enriched in n-3 fatty acids show the opposite results (Jensen et al., 1996; Minami et al., 1997; Suzuki et al., 1998; Wainwright et al., 1999; Lim & Suzuki, 2000; Petursdottir et al., 2002; Aid et al., 2003; Du et al, 2003; Champeil-Potokar et al., 2004). To date, there is minimal work being performed using AD transgenic mice to study effects of n-3 deficiency or supplementation. Hashimoto et al. (2002) did, however, experiment with rats infused with the A β ₁₋₄₀ peptide into the left ventricle to gain similar results that could be seen from an AD transgenic model. Rats infused through osmotic minipumps with the A β ₁₋₄₀ peptide for 3 weeks and supplemented with DHA for 12 weeks prior to A β infusion and continued for 3 weeks after infusion showed a decreased level of AA lipid content within blood plasma, the cortex, and the hippocampus compared to unsupplemented A β -infused rats. There was also a higher level of DHA lipid content

detected in blood plasma, the cortex and the hippocampus of the A β -infused group with DHA supplementation. The authors suggest that the decrease in AA for the DHA supplemented group could be due to a physical replacement of AA within blood plasma, the cortex, and the hippocampus by DHA or through an unexplained mechanism to decrease AA synthesis by increased DHA synthesis. The study also found significant positive correlations between cortical and plasma DHA levels as well as between hippocampal and plasma DHA levels. These two associations suggest a high degree of integration of DHA into the cortex and hippocampus from the plasma. Rats that were infused with the A β ₁₋₄₀ peptide not supplemented with DHA exhibited decreased plasma DHA versus vehicle rats (Hashimoto et al., 2002). This result is similar to that seen in patients with Alzheimer's disease where plasma levels of DHA are decreased compared to age-matched non-demented control subjects (Conquer et al., 2000; Tully et al., 2003).

A more recent study by Hashimoto et al. (2005) used rats fed a fish-oil deficient diet for 3 generations after which they were infused with either A β ₁₋₄₀ or vehicle. Rats from each group were administered either DHA or vehicle and fatty acid levels in the plasma, cerebral cortex and hippocampus were determined. The results were similar to those in the authors' previous work, such that there was an increase of DHA and a reduction in AA content in all three regions analyzed for groups administered DHA irrespective of A β infusion. In addition to fatty acid levels, the authors also measured oxidative stress in the cerebral cortex and hippocampus. TBARS and ROS levels were increased in the A β -infused group compared to the remaining three groups. These results suggest that infusion of A β ₁₋₄₀ is sufficient to produce oxidative damage to specific regions of the brain, so that administration of DHA reduced the levels of oxidative

damage to those of the vehicle group. The authors suggest that the reduction in AA content in the brain played a role in the ability for DHA to correct the oxidative damage generated by the infusion of A β ₁₋₄₀ (Hashimoto et al., 2005).

Recently, Calon et al. (2004) fed 17 month old non-transgenic (Tg-) and Tg2576 APP mice for ~3½ months a control diet, n-3 deficient safflower oil diet and n-3 deficient supplemented with DHA. Analysis of the frontal cortex of the APP mice showed that the n-3 deficient diet resulted in a decreased amount of DHA content compared to the control and DHA-supplemented diets. Also, the DHA-supplemented APP frontal cortex showed a decrease in the amount of AA compared to the n-3 deficient mice. Within the Tg- mice, the DHA-supplemented group had an increased amount of DHA content and decreased AA content in the frontal cortex versus the n-3 deficient Tg- mice.

Aside from frontal cortex fatty acid content, the study by Calon et al. (2004) also measured dendritic spine pathology. Fractin is a caspase-cleaved fragment of actin that is labeled within dendrites of tangle-bearing neurons. The fractin/actin ratio was measured in the cortex of aged (22 month) APP mice in comparison to age-matched non-transgenic mice. The APP mice had an increased ratio in the cortex however, the level of synaptophysin staining did not differ between the groups and the level of drebrin (dendritic spine actin-regulating protein), was decreased in APP mice. In comparison, temporal cortex from AD brains showed decreased content of drebrin and synaptophysin in comparison to control brains. To test the hypothesis that DHA directly plays a role in regulating caspase activity in the synapses of dendrites, Calon et al. (2004) measured the fractin/actin ratio in the cortex of APP and Tg- mice and found that DHA supplementation reversed the increased ratio of the control and n-3 deficient APP mice.

In order to determine if the caspase activity was induced by pre- or post-synaptic changes, drebrin (postsynaptic marker) was compared to SNAP-25 (presynaptic marker). The n-3 deficient APP mice exhibited a significant decrease of drebrin in the membrane fraction with an increase within the cytosolic fraction, leading the authors to conclude that drebrin is not a caspase substrate. The cytosolic increase of drebrin is consistent with its retention by intact F-actin filaments. The APP control and DHA-supplemented groups appeared to have opposite effects of drebrin versus the n-3 deficient diet group. The postsynaptic marker, SNAP-25 did not differ between any of the groups indicating that caspase activity is not altered postsynaptically by n-3 deficiency or DHA supplementation. In order to account for the presynaptic change associated with DHA supplementation, neuronal loss was examined. Consequently, no differences in neuronal number were found in cortex or hippocampus between APP or Tg- mice despite dietary intervention (Calon et al., 2004). Enrichment of mouse neuroblastoma cells with DHA showed a protection against apoptosis via phosphatidylinositol 3-kinase (PI3-K) (Akbar & Kim, 2002). In order to determine this effect in an AD mouse model, Calon et al. (2004) measured the levels of a protein subunit of PI3-K protein, p85 α . The n-3 deficient APP mice showed a significant reduction on p85 α in the cortex; DHA supplementation resulted in an increased amount of p85 α compared to the n-3 deficient mice, but still significantly less than the controls. From this, the authors suggest that n-3 depletion in APP animals disables the PI3-K pathway to prevent caspase activation which leads to postsynaptic pathology similar to that seen in AD subjects.

Calon et al. (2004) also investigated oxidative stress markers following n-3 depletion and DHA supplementation, as measured by dinitrophenylhydrazin (DNPH)

derivatized carbonyls in the cortex. The n-3 deficient group exhibited an increased amount of carbonyls compared to the APP control and DHA-supplemented groups. The authors conclude that caspase-mediated actin cleavage and loss of drebrin in an APP mouse model exhibiting AD pathology is regulated by n-3 PUFAs within the brain. Also, DHA, specifically, is involved in maintaining the PI3-K pathway to decrease apoptosis; however, there were no differences in neuronal number found between any of the diet groups.

Despite the lack of a DHA effect on the post-synaptic marker, SNAP-25, Calon et al. (2005) analyzed the effect of DHA on NMDA receptors within the same groups of APP transgenic mice. Both n-3 deficient and DHA supplemented Tg⁺ mice had reduced levels of NR2A, NR2B receptors, as well as reduced calcium/calmodulin-dependent kinase II (CaMKII) within the cortex compared to control Tg⁺ mice. Although, the DHA supplemented Tg⁺ mice had slightly elevated levels of NMDA receptor subtype 2A (NR2A), NMDA receptor subtype 2B (NR2B) and CaMKII, there was no significant difference between the dietary groups. However, the authors state that the addition of DHA resulted in a protective effect with respect to the loss of NR2A, NR2B and CaMKII (Calon et al., 2005). The authors offer no clear explanation as to how the n-3 depletion and DHA addition affected NMDA receptors. However, evidence shows that an increased number of NMDA receptors can lead to increased learning and memory in transgenic mice (Tang et al., 1999). Conversely, a reduction of NMDA receptor subtype 1 (NMDAR1) in the hippocampus of mice led to impairment of spatial learning (Tsien et al., 1996). The previous studies provide support for the improved memory that resulted from DHA supplementation as will be discussed in a later section. However, Lesné et al.

(2005) showed that activation of NMDA receptors in cell culture led to an increase of calcium influx, thereby activating CaMK and increasing production of A β peptides. Although the present study examined *in vitro* conditions, the implication exists that an alteration of NMDA receptor activation in a system that is prone to A β production will result in over-secretion of toxic A β peptides, ultimately leading to cognitive impairment (Lesné et al., 2005).

Lim et al. (2005) reported alterations of A β within 17 month old Tg2576 APP mice that were fed a control diet, n-3 deficient safflower oil diet and n-3 deficient supplemented with DHA for $\sim 3\frac{1}{2}$ months. The DHA supplemented mice had reduced levels of insoluble A β as compared to the n-3 deficient group, but there was no difference with respect to control mice. There were also no differences in soluble A β levels between any of the three groups. Despite the lack of effect of soluble A β , the DHA supplemented group had an overall reduction in A β plaque burden compared to the n-3 deficient mice. However, there was no comparison between the DHA supplemented group and the control group with respect to plaque burden. In addition, there was no difference in soluble A β , insoluble A β or A β_{40} between the DHA supplemented mice and the control APP mice, indicating minimal effects on A β by DHA.

Human Studies Involving Fatty Acids.

There have been numerous human trials examining fatty acid composition within blood or brain tissue of normal elderly and elderly subjects with dementia. Payet et al. (2004) performed a study using non-impaired elderly subjects to simply measure the

incorporation of fatty acids into erythrocyte membranes and plasma. DHA was supplemented for 9 months and resulted in a significant increase in total PUFAs within the blood mainly due to the increase of DHA and AA in the plasma and erythrocyte membranes. The observed increase of AA could have been the result of an increased amount of this fatty acid within the DHA supplementation diet compared to the control group's diet. There was no follow-up to indicate changes in cognition or everyday function (Payet et al., 2004). In a similar study, Boston et al. (2004) administered EPA for 12 weeks to AD patients. This study assessed cognitive improvement, as well as erythrocyte membrane composition of fatty acids both before and after treatment. At baseline, erythrocyte membrane fatty acid levels did not differ between AD and non-demented control patients. In short, the 12 week period of EPA administration was unsuccessful at altering any of the cognitive measures. However, treatment with EPA did result in significant increases in total n-3 fatty acids, specifically EPA and 22:5n-3 (DPA), compared to baseline levels (Boston et al., 2004).

In an earlier study, Tully et al. (2003) reported that low plasma levels of total n-3 PUFAs (specifically DHA) were associated with an increased risk of developing AD. This is in opposition to the above study by Boston et al. (2004) who found no difference in blood fatty acid levels between AD and non-demented subjects. The increased risk for AD with low plasma n-3's as reported by Tully et al. (2003) could be attributed to a lack of protection against cardiovascular disease, an increased production of pro-inflammatory cytokines, or a lack of nervous system homeostasis due to low plasma DHA and/or total n-3 PUFA content that would normally protect against these pathologies. Analysis of the MMSE scores of AD versus non-impaired subjects revealed that blood serum levels of

DHA and total saturated fat were significant predictors of AD (Tully et al., 2003). Engelhart et al. (2002a) found an association between risk of developing AD and an increased intake of total fat, saturated fat, transaturated fat and cholesterol. However, there were no significant associations between intake of total PUFA, n-6, or n-3 and the risk of AD (Engelhart et al., 2002a). Prior to much of the above information regarding the association between AD risk and n-3 intake/membrane content, Yehuda et al. (1996) showed that subjects with existing AD that were supplemented with SR-3 (which is a mixture of ALA and LA to yield a ratio of 4 to 1 of n-6 to n-3 fatty acids) had improved behavior, including enhanced short-term and long-term memory, more cooperation, better mood, increased appetite and more organization skills. This paper suggests that many of the improved symptoms offer benefit to the patient as well as the caregiver. The authors do not propose a mechanism of action for this compound, but suggest that changes in neuronal membranes due to SR-3 could lead to increased function of the neuronal system thereby yielding the above improvements (Yehuda et al., 1996).

Fatty acids, specifically DHA, rapidly incorporate into numerous different cell types, primarily into the phospholipids layers of their plasma membranes and mitochondria (Stillwell & Wassall, 2003). Most human AD studies involving analysis of fatty acids measure plasma levels or post-mortem brain tissue phospholipid levels. Early studies indicated that post-mortem brain tissue sections from subjects with AD or some other type of dementia had decreased levels of n-3 PUFAs, including ALA, DHA and EPA; decreases in n-6 PUFAs including AA and LA were also observed. In 1998, Corrigan et al. reported lower levels of total n-3 PUFAs, specifically decreased ALA in the parahippocampal phosphatidylcholine (PC) fractions from AD brains. The authors

suggest that the decrease in ALA is most likely due to a deficiency within the PC fraction to properly incorporate ALA into the phospholipid. Because no other PL fraction contained a significant decrease of ALA, the difference could not be accounted for by a change in dietary intake or liver synthesis of FAs between AD and control subjects. This study also found decreases in n-6 PUFAs, including AA, mainly within the phosphatidylethanolamine (PE) fraction of phospholipids of AD brains compared to age-matched controls (Corrigan et al., 1998). Prasad et al. (1998) also found a decreased level of AA in PE phospholipids, specifically within parahippocampal gyrus and the inferior parietal lobe of AD brain tissue compared to normal elderly brain tissue. DHA was only found to be significantly below normal levels within the PE fraction of the parahippocampal gyrus and PC fraction within the cerebellum. There were, however, decreased levels of total fatty acids in phospholipid classes within the parahippocampal gyrus and the inferior parietal lobe. Also measured were phospholipids within the superior and middle temporal gyri, where the only difference was a decrease of oleic acid in AD brain tissue in comparison to control brain tissue. This study also determined that there was a significant increase of senile plaque density within all three areas measured, parahippocampal gyrus, inferior parietal lobe and the superior and middle temporal gyri. The cerebellum contained only diffuse plaques. One of the possible links between the presence of senile plaques and the decreases of various fatty acids could be due to changes in the biosynthesis or degradation of the membrane phospholipids. The overall conclusion from Prasad et al. (1998) was that the changes in phospholipids content were due to the oxidative cascade present in AD brain tissue. Previous studies have indicated that the A β peptide is a prominent source of free radical generation. These free radicals

could be the source of oxidative damage to the phospholipids classes resulting in decreased levels of many of the fatty acids present in the membrane PLs. Additional mechanisms that could impact membrane PL composition are neuronal loss, changes in ion channel function or cellular biosynthesis mechanisms all due to the presence of A β plaques or NFTs (Prasad et al., 1998).

In contrast to both of the above studies, Skinner et al. (1993) found an increase in DHA in white matter of the parietal cortex from AD patients, in addition to an overall reduction in n-6 fatty acids in white matter of the frontal and parahippocampal cortices. However, this study also reported increases in adrenic acid (22:4n-6) in grey matter from the parietal, frontal and parahippocampal cortices, resulting in an increase of total n-6 fatty acids in the grey matter of the frontal cortex. The authors suggest that the changes in fatty acid composition between white and grey matter of the brain in AD patients could be due to an irregularity in the transportation of essential fatty acids to the brain (Skinner et al., 1993).

Recent evidence shows that not only brain tissue but plasma levels of fatty acids are different between AD subjects and normal elderly control subjects. AD subjects exhibit decreased plasma levels of DHA, EPA and total n-3 PUFAs in comparison to normal elderly controls (Conquer et al., 2000; Tully et al., 2003). Tully et al. (2003) determined no significant difference in total n-6 PUFA content in plasma levels; Conquer et al. (2000), however, found that AD subjects had a significantly lower n-3 to n-6 fatty acid ratio within plasma with a significant increase in the level of total n-6 PUFAs. The authors suggested that the mechanism by which these fatty acid levels become lower in AD subjects is simply a decrease in the dietary intake of these fatty acids (Conquer et al.,

2000). The authors also suggested that the lower levels could be due to an increase in the breakdown of DHA within the brain or a decreased amount within the plasma PC content which is the main source for plasma DHA. DHA content was not measured within the liver, therefore decreased liver synthesis of DHA could also account for the difference in plasma levels. Similar to Conquer, Heude et al. (2003) found a direct association between total n-6 fatty acids in erythrocyte membranes and risk of cognitive decline in a group of subjects during a 4 year study. Likewise, this study also determined that subjects with increased levels of n-3 fatty acids had a reduced risk of cognitive decline. However, this study did not include any information regarding dietary intake, therefore the reasons for the changes in fatty acid levels is unknown and could be due to alterations in liver metabolism, intake or transportation of essential fatty acids throughout the body.

Incorporating adequate amounts of omega-3 fatty acids into daily dietary intake is not plausible for the population as a whole. Metcalf et al. designed a study to survey healthy individuals for their n-3 consumption by providing foods enriched with n-3 PUFAs (2003). This study showed that people could consume adequate amounts of n-3 fatty acids in their diet to decrease plasma compositions of AA, LA and total n-6 fatty acids, as well as increase plasma levels of EPA, DHA and total n-3 fatty acids (Metcalf et al., 2003). However, trying to incorporate everyday foods with n-3 fatty acids will probably never occur. Kang et al. (2004) provided an alternative to enriching foods to obtain n-3 PUFAs. As previously mentioned, mammals can not convert n-6 fatty acids into n-3 fatty acids; however integrating a *fat-1* gene from *C. elegans* into a mouse allows this conversion to occur. Transgenic mice carrying this gene fed an n-3 deficient/n-6 rich diet had a higher percentage of n-3 fatty acids in tissues (muscle, heart, brain, liver,

kidney, lung and spleen) compared to non-transgenic mice fed the same diet. The n-6 to n-3 ratio within the transgenic mice was 1:1 whereas the non-transgenic ratio was 20-50:1 (Kang et al., 2004). This implies that dietary intake does not have to be altered in order to increase incorporation of n-3 fatty acids into tissues and plasma membranes. Yancy et al. (2004) and Stern et al. (2004) both show that a low fat diet results in less weight loss than a low carbohydrate diet at 6 months after dietary intervention. The low carbohydrate diet allowed for unlimited amounts of animal foods which implies increased protein and fat intake. This diet resulted in significantly more adverse side effects versus the low fat diet. These side effects included increased headaches, muscle cramps, diarrhea, general weakness and rash (Yancy et al., 2004). The low fat group was encouraged to decrease their fat intake to less than 30% of their daily energy. This group over one year lost the same amount of weight as the low carbohydrate group; neither group had any significant adverse side effects (Stern et al., 2004).

Neurochemical Effects of n-3 Fatty Acids.

Incorporation of fatty acids from fish oil into neuronal membranes has been shown to increase neuronal sensitivity to oxidation. Because of the high number of double bonds within DHA, it is more likely to be oxidized than other fatty acids, such as AA. Peroxidation of PUFAs can lead to damage to genes, membrane lipids and enzymatic proteins. Despite this, PUFA supplementation has been shown to correct the effects of oxidative stress by decreasing free radicals within the brain (Yehuda et al., 2002). Specifically, EPA can reduce reactive oxygen species (ROS) produced by lipid peroxidation by inhibiting the phospholipase A2 enzyme and stabilizing membrane

structure. Lipid peroxidation products can contribute to neurodegeneration by inhibiting glutamate or glucose transport within the brain. Supplementation with essential fatty acids for 30 days can decrease production of TBARS (end product of lipid peroxidation), xanthine oxidase (source of ROS) and nitric oxide (gaseous free radical) as well as increase superoxide dismutase (enzyme antioxidant) within the corpus striatum of adult rats (Sarsilmaz et al., 2003). In addition, enhanced activation of catalase and glutathione peroxidase, enzymes that decrease ROS, were evident in macrophages from ApoE-KO mice fed a diet supplemented with fish oil. These macrophages also had reduced levels of ROS and superoxide anion after treatment with fish oil back to normal levels (Wang et al., 2004). Increased production of lipid peroxides occurs in cerebellum and some cortical regions with age and in certain neurodegenerative disease, such as AD.

Specifically in AD, increased lipid peroxidation markers are detected within CSF, urine, plasma and the brain including all cortical lobes, hippocampus and cerebellum (Montine et al., 2004). The free radical induced peroxidation of AA yields a prostaglandin F₂-like compound named F₂-isoprostane (F₂-IsoP). Peroxidation of EPA produces F₃-IsoPs and peroxidation of DHA forms F₄-IsoPs also known as F₄-neuroprostanes (F₄-NP) because of their presence specifically within neurons (Roberts II et al., 1998; Nourooz-Zadeh et al., 1999). In some regions, brains of AD patients undergo significantly more lipid peroxidation than normal aging controls (Montine et al., 2004). Using an indicator that could reflect the amount of DHA oxidation within the brain could be used as a marker for detecting AD. Multiple studies have investigating the detection of DHA peroxidation within AD brains post-mortem and found significantly more F₄-NPs in occipital and temporal lobes in addition to the cerebral cortex (Nourooz-

Zadeh et al., 1999; Reich et al., 2001). This provides a sensitive indicator for oxidative damage to neurons because of the high concentration of DHA within the neurons. The peroxidation of DHA could be due to the outcome of the AD pathology or simply a predisposition to increase vulnerability to developing AD. However, following brain ischemia-reperfusion, DHA is released from the phospholipids membrane via PLA₂ and induces neuroprotection via suppression of pro-inflammatory cytokines, anti-apoptotic genes and formation of a novel docosanoid, NPD1 (Lukiw et al., 2005). NPD1 (10, 17S-docosatriene) is a bioactive DHA-derived lipid mediator. Lukiw et al. (2005) found that *in vitro* production of A β was attenuated with addition of DHA to the medium. The addition of DHA also produced an increase in NPD1 in these cultured cells, suggesting that the attenuation of A β peptide release could be partially due to the appearance of NPD1. The authors also determined a dose-dependent increase of NPD1 by sAPP α in conjunction with DHA, suggesting a positive feedback regulation between sAPP α and the DHA/NPD1 pathway to protect cells against neuronal damage. Lastly, Lukiw et al. (2005) found that NPD1 protected cultured cells from A β ₄₂-induced apoptosis by up-regulating anti-apoptotic genes, Bcl-xl, Bcl-2 and Bfl-1(A1).

Some research indicates that fish oil supplementation have anti-inflammatory actions, including decreases of some pro-inflammatory cytokines (IL-1 β , IL-2, IL-6, TNF- α) and increases of anti-inflammatory cytokines (IL-10, TGF- β). Few studies have focused on the effects of DHA and/or EPA specifically on inflammatory measures. Tomabe et al. (2000) showed that dietary DHA, but not EPA, supplementation resulted in a decrease of CD4-positive T lymphocytes to reduce ear swelling of mice with contact

hypersensitivity. In addition, DHA decreased the expression of pro-inflammatory IFN- γ , IL-6, IL-1 β and IL-2 mRNA within the ears (Tomobe et al., 2000).

In contrast to the lack of effect on inflammatory cytokines by EPA by Tomobe, Komatsu et al. (2003) found that a high dose of EPA inhibited nitric oxide (NO) production by LPS-activated peritoneal macrophages from mice. However, EPA did not have any effect on the inducible NO synthase (iNOS) protein. Treatment with DHA resulted in a significant inhibition of NO production and iNOS expression by murine peritoneal macrophages dose-dependently. The authors showed that the DHA treatment suppressed activation of the transcription factor nuclear factor (NF)- κ B most likely through inhibition of intracellular peroxides induced by IFN- γ and LPS, which lead to the suppression of NO production and iNOS expression. Within the macrophages, DHA treatment resulted in an up-regulation of intracellular glutathione (GSH); lowering GSH levels reversed the effects induced by DHA on NO production and NF- κ B. Therefore, the authors suggested that DHA inhibited NO production via suppression of NF- κ B and mediated by an up-regulation of GSH (Komatsu et al., 2003). Peritoneal macrophages from mice fed a control diet supplemented with menhaden fish oil for 15 weeks had decreased production of IL-1 β and TNF- α in addition to a reduction of mRNA for IL-1 β and TNF- α (Renier et al., 1993). Billiar et al. (1988) found an anti-inflammatory response exhibited by decreased IL-1 production by Kupffer cells of rats fed a fish oil supplemented diet for 6 weeks. N-3 PUFA supplementation, mainly DHA and EPA, in older women (51-68 years) for 3 months resulted in decreased production of all pro-inflammatory cytokines measured in the blood, including IL-1 β , TNF- α and IL-6

(Meydani et al., 1991). Specifically, IL-2 expression was reduced as a result of a decrease in helper-T cells, with fish oil supplementation leading to an anti-inflammatory result. In addition, there was an increase in the number of suppressor T cells detected that aid in the anti-inflammatory reaction by secreting IL-10 and/or TGF- β (Meydani et al., 1991).

In some contrast to the above studies, Puskas et al. (2004) reported a combination of pro-inflammatory and anti-inflammatory effects within mice enriched with fish oil and cholesterol. Puskas et al. (2004) fed mice a cholesterol-rich diet or a cholesterol-rich plus fish oil enriched diet for 2 months beginning at 4½ months of age. Within whole brain of mice fed the cholesterol-rich diet with fish oil, the pro-inflammatory cytokines (IL-6 and TNF- α) were down-regulated in addition to an anti-inflammatory cytokine, IL-10. Whereas within the retina, for the same group, an opposite result was seen, so that there was an up-regulation of TNF- α . The authors suggested that the eye was more sensitive to the high fat content of the diet and therefore and increased pro-inflammatory gene expression in which the addition of fish oil was not able to correct. However, the addition of the fish oil led to an overall anti-inflammatory result within the brains of mice fed a diet enriched with cholesterol plus fish oil, so the authors' suggestion is unclear. In addition, the positive effects expected by the fish oil could not be seen without including a group that was only administered fish oil without the addition of excess cholesterol. Without this group, it is difficult to draw conclusions without cholesterol confounding the results.

Additional studies find that n-3 supplementation results in net pro-inflammatory reactions. Lipopolysaccharide-stimulated macrophages from mice given fish oil

exhibited an increase in TNF- α secretion as well as a decrease of IL-10 secretion (Petursdottir et al., 2002). Liver phospholipid content showed a significant increase of n-3 PUFA content and a decrease of n-6 PUFA content in mice fed the fish oil supplement. The authors propose that the increased TNF- α secretion was mediated by a decrease in prostaglandin production. As discussed previously, AA and EPA can form prostaglandin intermediates; AA results in PGE₂ whereas EPA synthesis can lead to PGE₃ production. EPA and AA both compete for prostaglandin synthesis so that increased amounts of n-3 fatty acids (e.g. EPA) displace production of PGE₂ by production of PGE₃. However, PGE₃ is not as mitogenic or inflammatory as PGE₂ (Bagga et al., 2003). An additional study reported an inverse relation between PGE₂ production and TNF production in LPS-stimulated macrophages from mice fed a diet with a high n-3 to n-6 ratio (Watanabe et al., 1993). The authors suggested that PGE₂ was acting as a negative feedback effector on TNF- α production; decreased TNF- α production from n-3 enriched macrophages concomitantly with increased production of PGE₂. However, no established mechanism was suggested to explain the inverse relationship. It is therefore difficult to determine the mechanism responsible for this pro-inflammatory response from fish oils. In support of the findings by Petursdottir, Wallace et al. (2003) found that fish oil supplementation to healthy adults (18-39 years of age) resulted in a significant increase of IL-6 expression in blood mononuclear cells. There were no other changes evident in any of the other cytokines (TNF- α , IL-1 β , IL-2, IFN- γ) or inflammatory markers (T- and B-cell lymphocytes). The authors concluded that the dosage was not high enough to induce changes in additional cytokine production (Wallace et al., 2003). Overall, the mechanisms that PUFAs use to induce changes in the inflammatory cascade are

unknown, but it appears that dosages and duration of fish oil enrichment highly affect cytokine production positively and negatively.

DHA has also been shown to have effects on the cholinergic system. Increased aging results in decreased ACh levels in hippocampus, striatum (Ikegami et al., 1992), cingulate cortex (Baxter et al., 1999) and pyramidal neurons of the cerebral cortex (Casu et al., 2002); in addition there is an age-related decrease of DHA incorporation into membrane phospholipids within the whole brain, and specifically the hippocampus (Bacelo-Coblijn et al., 2003; Favreliere et al., 2003). The mechanism whereby DHA affects the cholinergic system is not well understood. A study by Jones et al. (1997) showed that an intravenous injection of a cholinergic agonist, arecoline, resulted in increased incorporation of DHA into membrane phospholipids and microsomal fractions within the rat brain after intravenous infusion of DHA. There was no change in response to an injection of saline or in combination with palmitic acid. The possible pathway involves the activation of phospholipase A₂ by arecoline to induce the release of DHA from the phospholipid PE, the main storage site of DHA (Jones et al., 1997). This suggests that the reduction in ACh levels leads to decreased DHA incorporation into neuronal membranes within the brain during aging.

Multiple studies illustrate the effect of DHA supplementation on the age-dependent dysfunction of the cholinergic system. PET scanning of the somatosensory cortex of aged monkeys fed a diet enriched with DHA showed a significant increase in their regional cerebral blood flow (rCBF) compared to control fed monkeys (Tsukada et al., 2000). Previous work has demonstrated that the cholinergic system induces an increase in rCBF so that the addition of a cholinergic antagonist resulted in decreased

rCBF. Overall, the authors propose that the change in the rCBF is due to the ability of DHA to modulate neuronal activation via the cholinergic system; specifically they speculate that DHA acts directly on cholinergic receptors to facilitate signal transduction and increase rCBF (Tsukada et al., 2000). Additional rodent studies also show that dietary supplementation of DHA for 3 months to aged (18 month) rats reverses an age-dependent cholinergic dysfunction so that basal ACh levels are similar to those of young rats (Favreliere et al., 2003). In stroke-prone spontaneously hypertensive rats, a significant increase in basal ACh levels in hippocampus and cortex is seen with the addition of DHA to the diet. Under control conditions these mice exhibit a dysfunction of the cholinergic system; DHA supplementation reversed that dysfunction (Minami et al., 1997). However, Aid et al. (2003) found that rats fed an n-3 deficient diet also increased basal ACh within the hippocampus compared to control fed rats. The authors suggested that this increase could be due to an increased release or decreased catabolism of ACh within the synaptic cleft due to changes in neuronal membrane composition which affects ion channels and therefore ACh levels (Aid et al., 2003). A diet rich in DHA results in increased KCL-induced release of ACh from the right ventral hippocampus in aged rats compared to aged rats on a control diet (Favreliere et al., 2003). The authors suggest that this release could be due to an increase of synaptic transmission or a decrease of membrane rigidity due to DHA incorporation into the plasma membrane. Similar to these results, an n-3 deficient diet results in decreased KCL-induced ACh release in hippocampus compared to control-fed rats (Aid et al., 2003).

Effects of n-3 Fatty Acids on 2nd Messengers.

N-3 and n-6 fatty acids have been shown to impact intracellular signaling, including second messenger systems. Briefly, within the visual transduction pathway, G protein (Gt) is activated by coupling to metarhodopsin (MII), resulting in closure of cGMP-gated channels within the rod outer segment (ROS) and induction of the neuronal response to light. Specifically, rhodopsin is photoactivated into MII by absorption of a photon of light and subsequently coupled to Gt. This coupling activates a cGMP-specific phosphodiesterase (PDE); hydrolysis of cGMP leads to closing of the cGMP-gated channels, changes in the transmembrane potential, and initiation of the neuronal response to light (Salem et al., 2001). MII formation has been determined to be a function of phospholipid acyl chain unsaturation; thus, DHA-enriched phospholipids from ROS membranes of rats raised on an n-3 adequate diet result in an increase of MII formation (Mitchell et al., 2003). Also, the rate of coupling of MII to Gt is increased in DHA-enriched bilayers compared to less unsaturated phospholipids. Conversely, the activity of PDE from ROS membranes of rats raised on an n-3 deficient diet was decreased (Mitchell et al., 2003). Also, the inclusion of cholesterol in ROS membrane bilayers results in a decrease of MII formation and PDE activation, in addition to a slower coupling of MII to Gt. The addition of cholesterol in conjunction with DHA, however, results in increases of MII formation and PDE activation as well as a faster coupling of MII to Gt (Litman et al., 2001). The authors conclude that DHA promotes optimal functioning of the G-protein coupled signaling pathway in the retina and suggest that a deficiency of n-3 fatty acids, specifically DHA, could lead to a decreased efficiency in related neurotransmitter functioning and the visual signaling pathway.

Cyclic AMP and GMP activation have also been implicated as being affected by DHA and/or EPA enrichment. Cultured neonatal rat ventricular myocytes were grown in a DHA- or EPA-enriched medium (Picq et al., 1996). Cultures from both fatty acids resulted in increased basal levels of cAMP and more prominently cGMP. Also, stimulation of cGMP was increased in n-3 enriched myocytes (Picq et al., 1996). Likewise, cultured rat myocardial cells have been studied to determine the impact of DHA or EPA incubation on adrenoceptor function (Grynberg et al., 1995). Incubation with either fatty acid resulted in similar n-6 to n-3 ratios within the cultured cells. However, the DHA-enriched cells showed a significantly higher stimulation of beta-adrenergic receptors with no change in the actual number of receptors. Also, DHA enrichment resulted in a decreased affinity of the beta-receptor for the ligand, dihydroalprenolol, and a decrease in beta-adrenergic induced cAMP production. To account for the enhanced stimulation of beta-adrenergic receptors and decrease of cAMP production, the authors incubated the myocardial cells with a permeant analogue of cAMP in conjunction with DHA; this resulted in a positive chronotropic response. Overall, they concluded that DHA enrichment of rat myocardial cells results in a positive effect on beta-adrenergic transduction via an increase of cAMP efficiency (Grynberg et al., 1995). In addition to the affect of DHA and/or EPA on cAMP and cGMP, activation of the cAMP pathway affects DHA release from rat brain astrocytes (Strokin et al., 2003). Because astrocytes are a source of DHA synthesis for the CNS, the study investigated the role of specific second messengers on DHA release for use in the CNS. Initially, addition of ATP to astrocytes stimulated release of DHA mediated by a Ca^{2+} -independent phospholipase A. Additional neurotransmitters which cause comparable

release of DHA were bradykinin, glutamate and thrombin. Adenylyl cyclase, protein kinase A (PKA) and cAMP all caused an increased release of DHA, which is blocked by inhibitors of adenylyl cyclase and PKA (Strokin et al., 2003). Despite the data to support the relationship between n-3 fatty acids and second messenger systems, such as cAMP and cGMP pathways, no strong evidence is shown to illustrate a mechanism causally linking fatty acids to these intracellular systems.

In addition to inducing effects on cAMP, DHA has also been shown to affect Ca^{2+} and Na^+ channels. PUFAs have previously been shown to inactivate voltage-gated sodium channels so that cardiac myocytes remain in a hyperpolarized state, similar to the effect seen by local anesthetics and anticonvulsant drugs (Xiao et al., 1995). Similarly, Vreudgenhil et al. (1996) showed that incubation of rat CA1 pyramidal neurons with either DHA or EPA resulted in a decrease in neuronal excitability via inactivation of voltage-gated sodium and calcium. Monounsaturated fatty acids, saturated fatty acids and LA were also incubated with the CA1 neuronal cells, but did not result in any change in neuronal excitability or sodium or calcium currents (Vreudgenhil et al., 1996). This implies that some specific mechanism is utilized by DHA and EPA to alter voltage-gated sodium and calcium channels. Bonin and Khan (2000) illustrate that DHA induces a mobilization of calcium from intracellular stores in the endoplasmic reticulum pool by the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Human (Jurkat) T-cells were cultured and incubated with either AA, EPA or DHA. DHA induced a dose-dependent calcium release intracellularly resulting in a spike with eventual return to baseline concentration; minimal response was seen with EPA and AA. DHA was also shown to cause an increase in intracellular calcium concentration, exhibited by refilling

of the ER pools. This effect was independent of IP₃, an intracellular messenger that facilitates the opening of calcium channels on the ER to refill the intracellular pools. However, the effect of DHA was found to be a result of increased influx of extracellular calcium through CRAC channels. Overall, DHA has the ability to influence intracellular calcium concentrations via CRAC channels and can therefore influence additional intracellular systems.

Gene expression can also be altered by the addition of a fish oil-rich diet. Fish oil supplementation to two-year old male Wistar rats for 1 month resulted in a significant increase in the expression of transthyretin (TTR) within the hippocampus (Puskas et al., 2003). Barcelo-Coblijn et al. (2003) also found an up-regulation of TTR in whole rat brains after supplementation with a fish oil-enriched diet. TTR is a thyroid hormone transport protein that is secreted by hepatocytes into the serum, and by the choroids plexus into the cerebral spinal fluid (CSF). Importantly, previous studies have shown that TTR inhibits aggregation of A β within the CSF (Schwarzman & Goldgaber, 1996) and binds to insoluble A β to prevent polymerization into plaques (Redondo et al., 2000). AD transgenic mouse models that exhibit A β plaques do not exhibit other neuropathologies observed in AD human subjects, such as neurofibrillary tangle formation or the neuronal loss characteristic in AD. However, APP transgenic mice express high levels of α -secretase cleaved APP (sAPP α) and TTR. However, infusion of an antibody, into the midscapular region, against TTR into the aged (18 month) APP mouse resulted in increased A β accumulation, tau phosphorylation, neuronal loss and apoptosis all within region CA1 (Stein et al., 2004). The authors concluded that TTR expression is protective in the APP transgenic mouse against some of the

neuropathologies present in AD human subjects, mainly A β deposition, neuronal loss and abnormal tau phosphorylation. Likewise, human AD subjects express lower levels of TTR compared to age-matched non-demented control subjects (Stein et al., 2004). Therefore, fish oil supplementation could induce an increase of TTR expression and in turn provide protective benefits against AD neuropathological progression.

In addition to alterations in TTR expression, changes in peroxisome proliferator-activator proteins (PPARs), sterol-regulatory element-binding proteins (SREBPs), and fatty acid binding proteins (FABPs) have been observed with fish oil supplementation. Puskas et al. (2004) fed 4½ month old mice for 2 months standard chow, a cholesterol-rich diet or a combined diet of high cholesterol plus fish oil. There was an up-regulation of PPAR- α and PPAR- γ within the whole brain of the mice fed the combined diet compared to standard-fed mice. However, there was also an up-regulation of PPAR- α in the brain of mice fed the cholesterol-rich diet as well. PPARs play an important role in regulating lipid and glucose metabolism and are activated by a diversity of ligands, specifically including PUFAs and fatty acid metabolites. PPAR- α has been shown to increase fatty acid catabolism, thereby having an overall lipid lowering effect (Schmitz & Langmann, 2005). This effect was most likely in response to the increased intake of cholesterol despite fish oil supplementation. However, PPAR- γ was up-regulated within the combined diet group only. PPAR- γ regulates genes that control cell proliferation and differentiation and is highly concentrated in adipocytes (Schmitz & Langmann, 2005). The authors offer no direct explanation for the changes in PPAR gene expression between the diets (Puskas et al., 2004). There was also an up-regulation of retinoid X receptor- γ (RXR- γ) in the whole brain of the mice fed the combined diet of cholesterol

and fish oil versus the standard diet. RXR- γ is increased during neuronal maturation, implicating an important role during development. In addition, RXR- γ is also implicated in working memory function related to the frontal and perirhinal cortices (Wietrzyk et al., 2005). However, Puskas et al. (2004) did not behaviorally evaluate these animals, thereby not determining if the increase in RXR- γ is sufficient to provide cognitive benefit.

SREBPs are essential in maintaining fatty acid and cholesterol homeostasis. Within the different isoforms, the main role for SREBP-1a is to regulate lipogenesis and cholesterol synthesis proteins, while SREBP-2 regulates genes involved in cholesterol metabolism. In Puskas et al (2004), mice fed the cholesterol-rich diet had increased expression of both SREBP-1a and SREBP-2 within whole brain and retina as compared to the standard fed mice. In contrast, the addition of the fish oil to the cholesterol diet returned these levels back to those found from the standard-fed mice. Therefore, the addition of the fish oil attenuated the affect induced by the high cholesterol within both brain and eye of these mice.

Lastly, Puskas et al. (2004) measured different forms of FABP within the brain and eye. Of the four types of FABPs measured (brain, epidermal, liver, and heart), the cholesterol-rich diet did not alter any of these proteins. However, addition of fish oil to the cholesterol-rich diet induced an increased expression of both epidermal FABP and heart FABP in both the whole brain and retina. Because FABPs regulate fatty acid content in various tissues (Verekamp & Zimmerman, 2001), the authors suggested that the FABPs compensated for the addition of the fish oil which caused an accumulation of fatty acids in the retina and the brain (Puskas et al., 2004).

Effects of n-3 Fatty Acids on LTP.

Long-term potentiation (LTP) has been associated with learning and memory functions. Increased aging is also associated with a decline of learning and memory and therefore also with an impairment of LTP. The mechanism involved in the age-dependent impairment of LTP is not known, however the possible mechanism could involve a decrease in the release of glutamate which normally assists in maintaining LTP within the perforant path of CA1 of the hippocampus (McGahon et al., 1999; Martin et al., 2002). As previously mentioned, increased aging is associated with a reduction of PUFA concentration within the neuronal membranes which leads to a decrease of membrane fluidity. This factor could contribute to the decreased release of glutamate and impaired LTP seen in aged individuals. Supplementation of PUFAs including AA+GLA, EPA (Martin et al., 2002) and DHA (McGahon et al., 1999) to aged rodents results in a reversal of the age-related impairment of LTP. Specifically, the effect by DHA is proposed to involve increasing membrane fluidity to affect neurotransmitter release (McGahon et al., 1999). Within the dentate gyrus (DG), DHA induced an excitatory effect on the excitatory post-synaptic potentials (EPSPs), but did not affect LTP within this region (Itokazu et al., 2000). The authors suggested that the effect of DHA on the EPSPs is mediated through potassium channels. DHA has previously been shown to block potassium channels which is abolished by the presence of zinc (Poling et al., 1995). Because zinc is more abundant within DG, the effect of DHA on potassium channels to cause a stimulatory effect on EPSPs should have been inhibited in DG. However, the authors' saw a stimulation of EPSPs with DHA. Thus, their explanation of results is flawed and is contradictory to the role of potassium channels as having inhibitory effects

on neurons. DHA, in combination with zinc in the DG, should have resulted in an overall inhibitory effect on EPSPs, not excitatory, if their explanation was correct. In contrast to the DG, within region CA1 the addition of DHA induced a dose-dependent inhibitory effect on EPSPs (Itokazu et al., 2000). Previous work has shown that DHA promotes the opening of NMDA receptors via a DHA-binding site on the receptor; however addition of an NMDA antagonist does not affect the EPSP in DG or CA1 resulting from supplementation with DHA (Itokazu et al., 2000). Young et al. (2000) also showed that DHA can act independent of the post-synaptic NMDA receptor. The authors suggest that DHA blocks sodium channels to stabilize presynaptic membranes at the resting membrane potential in order to decrease glutamate release which would lead to an inhibition of EPSPs (Young et al., 2000). Because of maintenance of the resting membrane potential, Ca^{2+} would not be released from intracellular stores to bind to glutamate autoreceptors, thereby decreasing a release of glutamate from the presynaptic cell. Overall, n-3s, specifically DHA, have been shown to have an inhibitory affect on EPSPs primarily through closures of sodium and potassium channels.

Behavioral Effects of n-3 Fatty Acids in Animals.

Numerous animal studies show associations between n-3 or n-6 intake and behavioral performance, as seen in Table 1 below. In reference to general locomotor activity, Carrie et al. (2000) showed that sardine oil supplementation for 2 generations resulted in an increase of activity in F2 young (7-11 weeks) mice, where adult (9-10 months) and aged (17-19 months) mice showed no difference with respect to palm oil fed animals. In contrast, Chalon et al. (1998) also found that fish oil supplementation for 2

generations decreased activity in young (2 month) rats. Within the same study, there were no differences in entries or time in open arm for the elevated plus maze task with young rats either fed a diet enriched with fish oil or control diet (Chalon et al., 1998). Rats that underwent surgery that occludes the common carotid arteries (2VO) had impaired spatial learning abilities. De Wilde et al. (2002) showed that n-3 PUFA administration from 3 weeks to 4 month of age to 2VO rats did not result in any differences seen in the elevated plus maze task at 5 months of age in comparison to vehicle control rats.

Changes in learning and memory performance are also associated with n-3 dietary intake. Ikemoto et al. (2001) weaned 1-month old rat pups onto either a diet supplemented with DHA or safflower oil from dams fed a safflower oil rich, n-3 deficient, chow. Between 11 and 18 weeks of age the rats were tested in a brightness discrimination task. Rats that received DHA supplementation performed similarly to rats not supplemented with DHA (Ikemoto et al., 2001). This shows that the cognitive impairment measured by a brightness discrimination task caused by an n-3 deficient diet is not reversible. Impairment present in stroke-prone spontaneously hypertensive (SHRSP) rats was also reversed for a passive avoidance test (Minami et al., 1997). In that study, SHRSP rats were given either a control diet with 0, 1 or 5% DHA beginning at 1½ months of age for 3½ months. SHRSP rats fed the DHA had increased latency (better memory) in passive avoidance testing compared to SHRSP rats fed 0% DHA (Minami et al., 1997). In another study, Carrie et al. (2000) showed that young rats (2-3 months) fed a diet enriched with sardine oil for 2 generations showed an increase in active avoidance performance compared to young control rats only on day 1 of the 5-day task, indicating a

lack of an overall effect of the sardine oil enrichment. There were also no differences in active avoidance performance between sardine oil supplemented and control diet groups composed of adult and aged rats (Carrie et al., 2000). Consistent with Carrie et al. (2000), de Wilde et al. (2002) found that adult (5 month) 2VO rats given dietary DHA and EPA since 1½ months of age showed no difference in active avoidance compared to non-supplemented and vehicle control rats. In another study, Hashimoto et al. (2002) administered a shuttle avoidance task, similar to active avoidance, to adult (9-month) rats that had been fed a DHA rich or control diet for 3 months followed by either sham surgery or infusion of the A β ₁₋₄₀ peptide into the left ventricle. The avoidance task was given 1, 2 and 3 weeks after the surgery. The A β -infused mice were impaired in this task compared to the vehicle infused mice. Notably, DHA administration significantly improved avoidance learning for both the sham and A β -infused groups compared to both groups with no DHA supplementation (Hashimoto et al., 2002).

Table 1. Summary of behavioral studies involving n-3 deficiency or supplementation to rodents.

| Study | Animal Model | Dietary Supplementation | Duration of Supplementation | Task | Results |
|------------------------------|------------------------|---------------------------------------|--|--------------------|--|
| Barcelo-Coblijn et al., 2003 | 2M and 24M rats | Fish oil or control | 1 M | Morris water maze | No effect on acquisition or retention |
| Calon et al., 2004 | Tg2576 APP and NT mice | n-3 deficient | supplemented with DHA 17M to 22M | Visible platform | No effect |
| | | | | Morris water maze | Tg+ DHA improved acquisition; no effect on retention |
| Carrie et al., 2000 | Mice | Sardine oil or Palm oil 2 generations | Young (2-3M) Mature (9-10M) Old (17-19M) | Open Field | Young sardine increased activity |
| | | | | Active Avoidance | No effect |
| | | | | Morris water maze | Mature sardine increased time in former platform quadrant above chance |
| Chalon et al., 1998 | Wistar rats | Fish oil or control 2 generations | 2M | Open Field | Fish oil decreased ambulation |
| | | | | Elevated plus maze | No effect |

Table 1 (Continued).

| Study | Animal Model | Dietary Supplementation | Duration of Supplementation | Task | Results |
|------------------------|--|--|---|---------------------------|--|
| De Wilde et al., 2002 | Bilateral carotid occlusion in 4M rats | n-3 PUFAs or control | 1M to 7M | Elevated plus maze at 5M | No effect |
| | | | | Active avoidance at 5M | No effect |
| | | | | Morris water maze at 7M | No effect on acquisition or retention |
| Gamoh et al., 2001 | Wistar rats | Fish oil deficient for 3 generations | DHA supplement from 24M to 26½M | 8-arm radial maze at 25½M | No effect |
| Hashimoto et al., 2005 | Aβ ₁₋₄₀ infused rats | Fish oil-deficient for 3 generations | DHA supplement from 8½-12M | 8-arm radial maze at 10½M | DHA had increased reference & working memory |
| Hashimoto et al., 2002 | Aβ ₁₋₄₀ infused rats | Fish oil-deficient 3 generations | DHA supplement from 6M to 9M | Shuttle avoidance at 9M | DHA had increased avoidance learning |
| Ikemoto et al., 2001 | Donryu rats | Safflower oil or Perilla oil (high n-3) 2 generations | 1M to 3M supplemented safflower diet with DHA | Brightness discrimination | No effect |
| Jensen et al., 1996 | Wistar rats | Seal oil; fish oil; vegetable oil; control for 4 generations | 3M | Morris water maze | No effect on acquisition or retention |
| Lim & Suzuki, 1999 | Young mice (1M); Aged mice (14M) | DHA or palm oil | 5 months supplementation | Maze learning -3 trials | DHA decreased errors & latency for selected trials |

Table 1 (Continued).

| Study | Animal Model | Dietary Supplementation | Duration of Supplementation | Task | Results |
|-------------------------|--------------------------|----------------------------|-----------------------------------|-------------------------|---|
| Lim & Suzuki, 2000 | Mice | DHA or palm oil | Beginning at 3M for ¼, ½, 1 or 3M | Maze learning -3 trials | DHA decreased errors & latency for selected trials |
| Minami et al., 1997 | SHRSP rats | 0, 1, 5%DHA | 1½ M to 5M | Passive avoidance | SHRSP+DHA had increased avoidance learning |
| Sugimoto et al., 2002 | Mice | DHA or control | 9M to 11M | 8-arm radial maze | DHA increased working memory |
| Suzuki et al., 1998 | mice | Sardine oil or palm oil | Beginning at ¾M for 12M | Maze learning -3 trials | Sardine oil decreased errors/latency for initial trials |
| Wainwright et al., 1999 | Artificially reared rats | 0-2.5% DHA/AA combinations | Postnatal day 5 to 1½M | Morris water maze | No effect on acquisition or retention |

Abbreviations: M, month; NT, non-transgenic mouse; Tg+, APP transgenic mouse; DHA, docosahexaenoic acid; PUFAs, polyunsaturated fatty acids; SHRSP, stroke-prone spontaneously hypertensive rats; AA, arachidonic acid.

Aside from avoidance tasks, spatial maze learning is a good indicator of both reference and working memory. Sugimoto et al. (2002) found a decrease in working memory errors in the eight-arm radial maze for adult (9 months) mice supplemented with a DHA-rich diet for 2 months compared to unsupplemented control mice; there was, however, no difference in reference memory errors. Gamoh et al. (2001) showed no overall improvement in performance in the same task with aged rats administered DHA from 24 months to 26½ months of age after two generations on a fish oil-deficient diet. Eight-arm radial maze learning, assessed by 3 trials per day for 3 separate days, beginning around 25 months of age for 5 weeks showed no difference in working or reference memory with DHA supplementation compared to rats without this supplement (Gamoh et al., 2001). More recently, Hashimoto et al. (2005) fed a fish oil-deficient diet to rats for 3 generations. At 5 months of age, the F3 generation rats were either infused through osmotic minipumps with A β ₁₋₄₀ or vehicle alone for 5 weeks. Subsequently, rats from each group were administered either oral DHA or vehicle for 7 weeks and then tested in the eight-arm radial maze for 5 weeks. Overall, DHA administration improved reference and working memory in both vehicle and A β -infused groups (Hashimoto et al., 2005).

Utilizing a different task involving maze learning, Suzuki et al. (1998) supplemented 3-week old mice for 12 months with sardine oil. Maze learning was assessed by 3 trials administered 4 days apart using a simple maze construction with one entry/exit and many blind alleys. Suzuki et al. (1998) found decreased latency and errors for trials 1 and 2; by trial 3 the palm oil fed mice exhibited a similar performance. A similar study by Lim and Suzuki (1999) fed DHA-enriched or palm oil-enriched diets to

3-month old and 14-month old mice for 5 months, followed by 3 trials of maze learning with the same protocol used by Suzuki et al. (1998). The young mice supplemented with DHA showed decreased latency and errors only on trial 2, whereas the aged mice supplemented with DHA showed decreased latency and errors on trials 2 and 3 (Lim & Suzuki, 1999). Similar to the above maze studies, young mice (3 months old) were fed DHA enriched diet or palm oil enriched diet for 1 week, 2 weeks, 1 month, or 3 months then tested for the same maze learning as previously discussed (Lim & Suzuki, 2000). The mice fed the DHA enriched diet for 1 and 3 months had decreased errors and latency only in trial 3 compared to palm oil enriched mice (Lim & Suzuki, 2000). The above results, performed with DHA administration for different durations, suggest that n-3 enrichment can improve cognitive performance at any age, although the data are far from conclusive.

A major task used to evaluate spatial learning/ memory among rodents is the Morris water maze. This task provides measures for reference learning during acquisition and memory retention during the probe trial. Jensen et al. (1996) showed that rats fed fish, seal, or vegetable oil-rich diets for 4 generations had a trend toward improved performance in the Morris water maze versus rats fed a control diet; however there were no differences between the groups in cognitive performance. The fish oil group had an insignificant decrease in acquisition latency at 3 months of age during the 9-day task with no overall difference within any of the groups (Jensen et al., 1996). In conjunction with the lack of effect from fish oil supplementation by Jensen et al. (1996), Barcelo-Coblijn et al. (2003) found that 3-month old rats that had been fed a diet enriched with fish oil for 1 month showed no overall differences in Morris water maze

performance (acquisition or retention) between the enriched versus control groups.

Within the same study, there was also no difference in Morris maze acquisition or retention performance in 24 month old rats fed a fish oil supplement for 1 month versus control fed rats (Barcelo-Coblijn et al., 2003).

In another study, artificially reared rats were fed milk supplements with 0 to 2.5% DHA and/or AA in combination from postnatal day 5 to postnatal day 18 (Wainwright et al., 1999). After postnatal day 18, rats were weaned onto diets that contained identical amounts of DHA and AA compared to the milk substitutes. At 1½ months of age, rats supplemented with DHA and/or AA showed similar latencies to controls during Morris water maze acquisition and retention testing; thus, no effect of DHA and/or AA was observed (Wainwright et al., 1999). In addition to open field and active avoidance, Carrie et al. (2000) used the Morris water maze to evaluate cognition in young (2-3 months), mature (9-10 months) or old (17-19 months) mice administered fish oil supplementation (sardine oil) or a palm oil enriched diet over two generations. This study found no difference in acquisition between any of the groups; however, only mature mice fed the sardine oil diet showed significant memory retention during the probe trial, as indicated by their spending more time than chance in the former platform-containing quadrant (Carrie et al., 2000).

Clearly, Morris water maze acquisition and retention cognitive performance does not seem be strongly affected by generations of fatty acid-supplemented diets in non-transgenic mice. Likewise, 17-month old Tg2576 APP transgenic and non-transgenic mice fed an n-3 deficient diet with or without DHA enrichment for ~3½ months showed no differences in latency in visible platform testing (Calon et al., 2004). Within the first

3 blocks of visible platform testing, the non-transgenic mice supplemented with DHA showed improved performance, as exhibited by a decreased latency compared to the non-transgenic mice on a low DHA diet. During Morris water maze testing, in the same study, APP mice fed the n-3 deficient diet showed impaired spatial learning only over the last two blocks of testing, days 7-9 and 10-12, compared to APP mice supplemented with DHA and non-transgenic mice with or without DHA enrichment. Although there was no overall effect of DHA supplementation on spatial learning, the authors conclude that DHA supplementation to the n-3 deficient APP mice corrected the learning impairment caused by n-3 deficiency. However, the probe trial measuring memory retention showed a lack of retention by all groups except the non-transgenic group supplemented with DHA that exhibited a partial quadrant preference. This group spent significantly more time in the quadrant that formerly contained the platform versus the opposite quadrant. No other group showed this preference, indicating that DHA supplementation could not correct the spatial memory deficits by the APP mice fed the n-3 deficient diet (Calon et al., 2004). Overall, n-3 supplementation appeared to impact avoidance learning more than spatial memory tasks such as the Morris water maze, possibly due to a difference in cognitive domains used between the tasks.

In conclusion, omega-3 fatty acids have been shown to have some beneficial affects on membrane lipid composition, inflammation, the cholinergic system and LTP. Also described were improvements of cognition in some human trials and animal studies. However, there are some drawbacks to the prior behavioral studies involving n-3 and n-6 fatty acids in human and animal studies. To date, there have been no human studies done that have used DHA and EPA in combination to analyze effects on AD as treatment or

prevention. Yehuda et al. (1996) did test the behavioral effects of LA and ALA together in AD patients over a short one-month treatment period; however, only about 15% of ALA is converted to DHA for use in the body. No other study has followed a treatment-based approach using fatty acids or fish oil to reverse cognitive decline due to Alzheimer's disease. However, essentially all of the "protection-based" human studies have been epidemiologic, retrospective studies which are often inaccurate and unable to control for many other factors that could affect cognition later in life. Additionally, there are no longitudinal studies that have administered or retrospectively looked at n-3 fatty acids or fish oil supplements to determine any beneficial behavioral affect. Aside from human studies, there are no animal studies involving an AD transgenic mouse model which utilized DHA and EPA as prevention therapy. Calon et al. (2004) used APP transgenic mice on an n-3 deficient diet supplemented with only DHA as treatment for AD. This approach was also used many times in non-transgenic rodent studies, as previously discussed, and is not a realistic approach to human dietary intervention. A more realistic approach would be a supplementation with an appropriate ratio of n-6 and n-3 fatty acids (composed primarily of DHA and EPA) that could be duplicated for human dietary prevention of AD. The majority of control diets that were used in animal studies were n-3 deficient, having only ALA as the primary n-3 fatty acid, and had no DHA or EPA. Also, the experimental diets used for comparison to the control diets do not incorporate both DHA and EPA specifically; instead they used an ethyl ester form of DHA or a complete fish oil supplement which adds additional n-3 fatty acids. Neither of these diet compositions accurately displays a sufficient human dietary intervention that would incorporate amounts of n-3 fatty acids present in foods. In addition, most of the

animal studies used only one behavioral task, either active avoidance or Morris water maze, to evaluate the behavioral affects of n-3 administration. Such behavioral analyses do not provide a complete assessment of cognitive changes that could occur from a dietary intervention study. Instead, a more complete behavioral analysis should include tasks that test multiple sensorimotor and cognitive domains.

XI. Statement of Purpose

As detailed in the Background section, two potential therapeutics against AD are A β -based immunotherapy and n-3 fatty acid administration. This proposal will involve studies that directly test the ability of these two therapies to protect against AD-like behavioral impairment and pathology in a transgenic mouse model for AD — the APP+PS1 mouse.

Regarding A β immunotherapy, to date there have been no studies that show life-long or longitudinal behavioral benefits of A β vaccinations. Also, no studies have evaluated the same vaccinated transgenic animals in an extensive behavioral battery that incorporates multiple sensorimotor and cognitive domains at several time points during the vaccination period. Therefore, this proposal involves long-term prevention-based A β immunotherapy to APP+PS1 mice that will:

- longitudinally evaluate cognitive function in at two separate time points during aging in an extensive behavioral battery.
- determine extent of A β pathology at the latter time point and correlate with behavior.

Regarding n-3 fatty acid administration, there have been no clinical studies performed to determine the protective or treatment effects of DHA+EPA supplementation against AD. Also, there have been no animal studies that have used a comprehensive behavioral battery to assess the effects of n-3 fatty acids, specifically DHA and EPA, on behavior in non-transgenic or AD transgenic animal models. Therefore, this proposal will administer to APP+PS1 mice a prevention-based high n-3 fatty acid supplement, rich in DHA and EPA. This diet is modeled to mimic a twice-weekly dietary intake of fish for humans. In addition, a high n-6 fatty acid diet, rich in LA, will be given to other APP+PS1 mice. This n-6 diet is modeled to mimic a typical (bad) Western diet. To compare the dietary effects of a high n-3 fatty acid diet versus a high n-6 fatty acid diet in APP+PS1 mice and non-transgenic mice, this proposal will:

- evaluate cognitive function in an extensive behavioral battery at 4 to 5½ months into dietary treatment.
- examine fatty acid levels in multiple tissues including brain, liver and plasma to note differential incorporation rates into membranes, which may potentially affect intracellular signaling.
- analyze markers that could impact AD progression and be affected by n-3/n-6 dietary manipulation, such as A β pathology, oxidative damage and inflammatory proteins, which correlation to behavior.

Materials and Methods

I. Life-long Vaccination Study

Animals. Double transgenic APP + PS1 mice were derived from a cross of the mutant APP_{K670N,M671L} transgenic line with the mutant PS1 transgenic line 5.1. APP mice had a C57B6/SJL X C57B6 background and PS1-5.1 mice had a Swiss Webster/B6D2F1 X B6D2F1 background, providing progeny with a mixed background. Non-transgenic littermates were used as controls, with all mice being from the eighth or ninth generation following the initial cross. After weaning, mice were genotyped and group housed until the start of vaccinations at two months of age. All mice were maintained on a 12 hour light-dark cycle, with free access to rodent chow and water. Behavioral testing was performed in the light period and in the same room where animals are being housed.

General Protocol. The general protocol for this study is temporally depicted in Fig. 3. Beginning at 2 months of age, APP+PS1 mice were injected monthly with the A β ₁₋₄₂ peptide (n=8) or phosphate-buffered saline (PBS) with adjuvant (n=6). Control non-transgenic littermates (n=9) received monthly injections of PBS with adjuvant. All mice were tested in a 6-week behavioral battery at 4½-6 months of age (2½-4 months into immunotherapy), then again in the same test battery at 15-16½ months of age (13-14½ months into immunotherapy). The test battery consisted of three sensorimotor-based, one anxiety-based, and five cognitive-based tasks, that were performed in the

following order: open field activity, balance beam, string agility, Y-maze, elevated plus maze, Morris water maze, circular platform, platform recognition, and radial arm water maze.

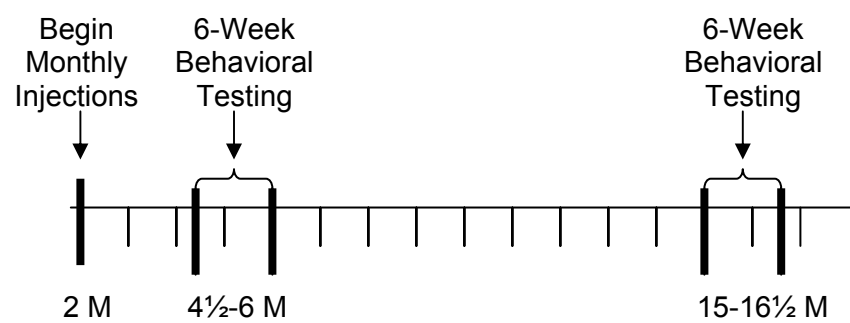


Fig. 3. General protocol time line for life-long vaccination study.

Following completion of the test battery at 16½ months of age, all animals were euthanized, their brain removed and processed for A β histopathology. Using DNA extracted from tail samples, PCR genotyping of animals for mutant APP and PS1 genes was performed at weaning, and again (for confirmation) on the day of euthanization. Procedures for all of the above were reviewed and approved by the USF Institutional Animal Care and Use Committee (IACUC).

Vaccination protocol. The human A β ₄₂ peptide (Bachem) was first suspended in pyrogen-free Type I water at 2.2 mg ml⁻¹ and then mixed with 10X PBS in order to yield 1X PBS. This solution was then incubated overnight at 37°C, then mixed with Freund's complete adjuvant at 1:1; 100 µg of the A β vaccination mix was injected subcutaneously into APP+PS1 mice. Each boost vaccination at monthly intervals was prepared fresh with incomplete Freund's. Injections for control APP+PS1 and non-transgenic mice consisted of PBS plus adjuvant prepared in the same way. The experimenter that performed these injections had no role in the behavioral testing of mice.

Histopathology and Image Analysis. Following completion of behavioral testing, mice were overdosed with pentobarbital (100 mg/kg *i.p.*) and perfused transcardially with 25ml of normal saline (0.9%). Brains were removed and immersion fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) for 24 hours; they were then cryoprotected in a series of sucrose solutions, frozen and sectioned in the horizontal plane at 25 µm using a microtome. Brain sections were stored at 4°C in Dulbecco's PBS for

A β immunocytochemistry (total A β = both A β 1-40 & A β 1-42) and Congo red (A β 1-40) histologic staining for compact deposition as described in a latter section.

II. Omega-3 Fatty Acid Study- Survival Analysis

Animals. All APP/PS1 and PS1 transgenic mice were obtained from a cross between heterozygous male mice carrying the mutant APP_{K670N,M671L} gene and homozygous female mice with the mutant PS1 transgenic line 6.2. All of the offspring contained a mixed background of 37.5% C57, 25% B6, 12.5% SJL and 25% Swiss Webster. The non-transgenic and APP single transgenic mice were obtained from a cross of the F1 PS1 female mice with P (parental generation) heterozygous APP male mice to obtain APP+PS1, APP, PS1 and non-transgenic (Tg-) offspring with a 56.25% C57, 12.5% B6, 18.75% SJL and 12.5% Swiss Webster mixed background. The mice were genotyped and singly housed after weaning.

General Protocol. Thirty-eight F1 APP+PS1 and twenty-seven F2 Tg- mice were started on either a low omega-3, omega-3-deficient diet (omega-6 only), or standard rodent chow at an average of 6 weeks of age (range = 4-8 weeks) (Fig. 4). The low omega-3 diet contained 13 APP/PS1 and 9 Tg- mice; there were 15 APP/PS1 mice and 9 Tg- mice in the omega-6 only diet group and the standard diet included 10 APP/PS1 and 9 Tg- mice. There was a 100% mortality rate for both Tg+ and Tg- mice on the omega-3 deficient diet, with most Tg+ mice dying between 20-25 days after diet initiation and all Tg- mice dying within 2-8 days of diet initiation. Because of the longer survival of the Tg+ mice on the omega-3 deficient diet, Study 1 has been designed to investigate the

potential role of APPsw and/or PS1 transgenes in protecting against early mortality that otherwise occurred in Tg- mice. Thus, 7 APPsw and 7 PS1 transgenic mice will be started on the omega-3 deficient diet at 2 months of age, with daily monitoring for mortality. To determine the effects of age on the mortality of APPsw+PS1 mice resulting from the omega-3 deficient diet, 2-3 APPsw+PS1 mice will be started on the omega-3 deficient diet at 2, 3 and 4 months of age. Daily monitoring for mortality will occur. Figure 2 indicates a timeline of when all Tg+ and Tg- mice were or will be started on the omega-3 deficient diet. Table 1 gives all of the major fatty acids present within the three diets. None of these animals will be behaviorally tested; they will only be monitored for health problems and rate of mortality once they are started on their respective diets.

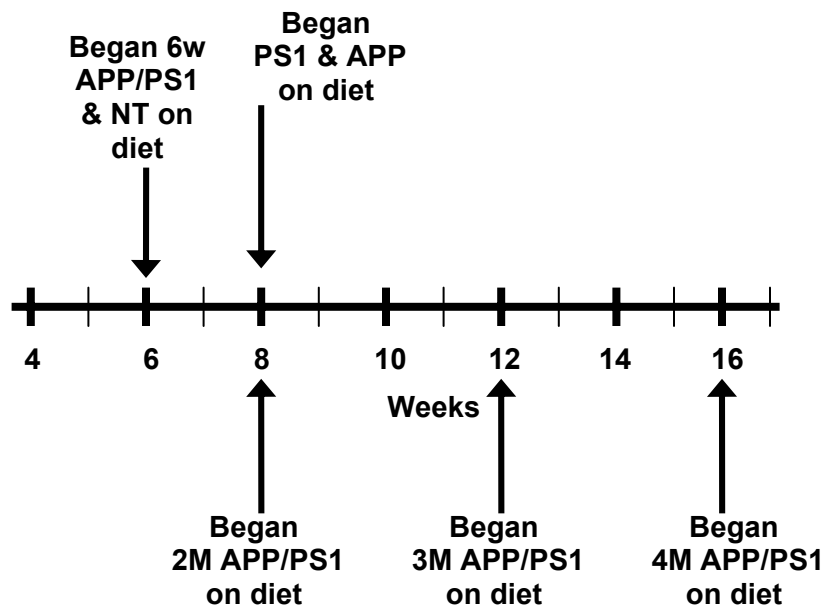


Fig. 4. General protocol time line for omega-3 fatty acid study-Survival Analysis.

Fatty Acid Diets. Both experimental diets were obtained from Purina Mills Test Diets (Lafayette, IN) and lipid profiles were performed by Barrow-Agee Laboratories to confirm the fatty acid content for each diet. The 2018 Teklad Global 18% Protein Rodent Diet standard diet used was obtained from Harlan. The low omega-3 fatty acid diet included 8.55% fat with the majority of fat from oleic acid and palmitic acid (Table 2). The n-6/n-3 ratio for this diet was 6.3 to 1. The omega-3-deficient diet included 6.72% fat with the majority of fat also from oleic acid and palmitic acid (Table 2). This diet contained an n-6/n-3 ratio which could not be determined because it contained no detectable amounts of any n-3 fatty acids. There were no detectable amounts of DHA or EPA in any of the three diets presented in Table 1. There were similar amounts of n-6 fatty acids present in both diets. The major fatty acids present in each diet are presented in Table 2.

Table 2. Percent fatty acid composition of total fat for three diets.

| | | Standard Diet | Low Omega-3 Diet | Omega-3-Deficient Diet |
|---|------------------|----------------------|-------------------------|-------------------------------|
| % Total Fat | | 5.00 | 8.55 | 6.72 |
| Total Saturated Fats | | 9.59 | 42.07 | 30.23 |
| Total Monounsaturated Fats | | 12.84 | 32.30 | 28.46 |
| Total Polyunsaturated Fats | | 34.11 | 8.42 | 4.64 |
| Saturated Fats | | | | |
| Butyric | C4:0 | 0.00 | 0.00 | 0.00 |
| Caproic | C6:0 | 0.00 | 0.00 | 0.00 |
| Caprylic | C8:0 | 0.00 | 0.34 | 1.16 |
| Capric | C10:0 | 0.00 | 0.00 | 0.00 |
| Lauric | C12:0 | 0.26 | 0.19 | 0.00 |
| Myristic | C14:0 | 0.06 | 8.19 | 1.99 |
| Pentadecanoic | C15:0 | 0.00 | 0.69 | 0.22 |
| Palmitic | C16:0 | 7.64 | 25.40 | 19.03 |
| Magaric | C17:0 | 0.00 | 0.67 | 0.00 |
| Stearic | C18:0 | 1.50 | 5.80 | 6.61 |
| Arachidic | C20:0 | 0.01 | 0.48 | 0.73 |
| Behenic | C22:0 | 0.03 | 0.316 | 0.50 |
| Lignoceric | C24:0 | 0.00 | 0.00 | 0.00 |
| Monounsaturated Fats | | | | |
| Palmitoleic | C16:1n-7 | 0.07 | 6.99 | 1.39 |
| Heptadecenoic | C17:1n-8 | 0.00 | 0.39 | 0.00 |
| Oleic | C18:1n-9c | 12.59 | 19.61 | 24.77 |
| Gadoleic | C20:1n-9 | 0.17 | 0.00 | 0.00 |
| Erucic | C22:1n-9 | 0.01 | 0.00 | 0.00 |
| Polyunsaturated Fats | | | | |
| Linoleic | C18:2n-6c | 31.35 | 7.27 | 4.64 |
| α-Linolenic | C18:3n-3c | 2.76 | 0.00 | 0.00 |
| γ-Linoleic | C18:3n-6c | 0.00 | 0.00 | 0.00 |
| Octadecatetraenoic | C18:4n-3 | 0.00 | 0.00 | 0.00 |
| Eicosadienoic | C20:2n-6 | 0.00 | 0.00 | 0.00 |
| Homo-γ Linolenic | C20:3n-6c | 0.00 | 0.00 | 0.00 |
| Arachidonic | C20:4n-6c | 0.00 | 0.00 | 0.00 |
| Eicosapentaenoic | C20:5n-3c | 0.00 | 0.00 | 0.00 |
| Docosapentaenoic | C22:5n-3 | 0.00 | 1.15 | 0.00 |
| Docosahexaenoic | C22:6n-3c | 0.00 | 0.00 | 0.00 |
| Total n-3 Fats | | 2.76 | 1.15 | 0.00 |
| Total n-6 Fats | | 31.35 | 7.27 | 4.64 |
| n-6/n-3 ratio | | 11.4 to 1 | 6.3 to 1 | ∞ |

II. Omega-3 Fatty Acid Study- Behavioral, Pathologic and Neurochemical Assessments

Animals. All mice were obtained from a second generation cross between heterozygous APP_{K670N,M671L} and heterozygous PS1transgenic line 6.2. The backgrounds of all offspring were a mix of C57/B6/SJL/Swiss Webster. The mice were genotyped and singly housed after weaning and a confirmatory genotyping was performed 1 month prior to behavioral testing. All mice were maintained on a 14 hour light and 10 hour dark cycle for the duration of the study; all behavioral testing was performed during the light cycle.

General Protocol. A total of 16 double transgenic APP+PS1 mice, 7 APP mice and 24 non-transgenic (NT) littermates were randomly divided into one of three diet groups. Beginning at 2 months of age, NT and transgenic (Tg+) mice were divided into three separate diet groups: high omega-3, high omega-6, and standard diets (Fig. 5). Because of the oxidative properties of omega-3 fatty acids the diets were stored at -20°C in 4-7 day supply aliquots. Mice fed the high omega-3 diet consisted of 7 APP+PS1 mice and 2 APP mice and 8 NT mice; the standard diet fed mice included 9 APP+PS1 and 1 APP mouse and 9 NT mice; the high omega-6 fatty acid diet group consisted of 1 APP+PS1 mouse and 4 APP mice and 7 NT. At 6 months of age, the NT groups were behaviorally tested in a 6-week battery that included three sensorimotor-based, one anxiety-based, and five cognitive-based tasks, performed in the following order: open field activity, balance beam, string agility, Y-maze, elevated plus maze, Morris water maze, circular platform, platform recognition, and radial arm water maze. At 7.5 months of age, Tg+ mice were examined in the same 6-week battery of tasks (Fig. 5). NT and

Tg⁺ mice could not be tested concurrently because of the large total number of animals involved (e.g., 47 mice). Immediately following behavioral testing all mice were euthanized; brains were then removed for A β histological and fatty acid analyses.

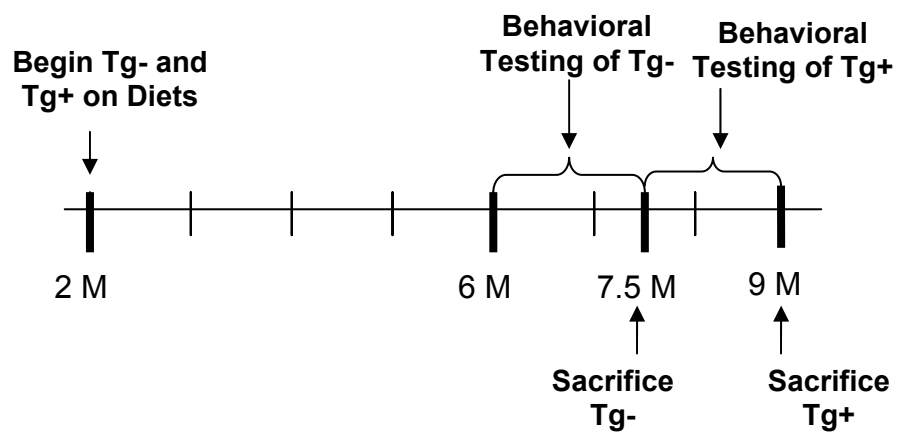


Fig. 5. General protocol time line for omega-3 fatty acid study II-Behavioral, Pathologic and Neurochemical Assessments

Fatty Acid Diets. Fatty acid diets were obtained from Purina Mills Test Diets (Lafayette, IN). Lipid profiles were performed by Barrow-Agee Laboratories for each diet prior to administration to the mice to confirm their fatty acid content. Each of the experimental diets contained 10% fat. The high omega-3 diet included 6% safflower oil and 4% menhaden fish oil with an n-6:n-3 ratio of 3.8 to 1; the high omega-6 diet included 9.5% safflower oil and only 0.5% menhaden fish oil resulting in an n-6:n-3 ratio of 32.8 to 1. The standard diet was the 2018 Teklad Global 18% Protein Rodent Diet received from Harlan, which was normally given to all mice within our mating colonies who were not on a special diet. As indicated in Table 3, this Harlan diet had only half the fat as the other two diets. The exact lipid composition for the major fatty acids present in each diet is indicated in Table 3.

Table 3. Percent fatty acid composition of total fat for three diets.

| | | Standard Diet | High Omega-3 Diet | High Omega-6 Diet |
|---|------------------|----------------------|--------------------------|--------------------------|
| % Total Fat | | 5.00 | 10.44 | 9.99 |
| Total Saturated Fats | | 9.59 | 20.04 | 10.79 |
| Total Monounsaturated Fats | | 12.84 | 19.73 | 14.59 |
| Total Polyunsaturated Fats | | 34.11 | 64.22 | 74.27 |
| Saturated Fats | | | | |
| Butyric | C4:0 | 0.00 | 0.00 | 0.00 |
| Caproic | C6:0 | 0.00 | 0.00 | 0.00 |
| Caprylic | C8:0 | 0.00 | 0.00 | 0.00 |
| Capric | C10:0 | 0.00 | 0.00 | 0.00 |
| Lauric | C12:0 | 0.26 | 0.06 | 0.00 |
| Myristic | C14:0 | 0.06 | 3.37 | 0.58 |
| Pentadecanoic | C15:0 | 0.00 | 0.28 | 0.06 |
| Palmitic | C16:0 | 7.64 | 11.88 | 7.54 |
| Magaric | C17:0 | 0.00 | 0.29 | 0.07 |
| Stearic | C18:0 | 1.50 | 2.84 | 2.16 |
| Arachidic | C20:0 | 0.010 | 0.28 | 0.33 |
| Behenic | C22:0 | 0.03 | 0.00 | 0.00 |
| Lignoceric | C24:0 | 0.00 | 0.09 | 0.11 |
| Monounsaturated Fats | | | | |
| Palmitoleic | C16:1n-7 | 0.07 | 4.03 | 0.60 |
| Heptadecenoic | C17:1n-8 | 0.00 | 0.29 | 0.08 |
| Oleic | C18:1n-9c | 12.59 | 11.94 | 13.03 |
| Gadoleic | C20:1n-9 | 0.17 | 0.094 | 0.24 |
| Erucic | C22:1n-9 | 0.01 | 0.07 | 0.00 |
| Polyunsaturated Fats | | | | |
| Linoleic | C18:2n-6c | 31.35 | 49.99 | 71.99 |
| α-Linolenic | C18:3n-3c | 2.76 | 0.70 | 0.13 |
| γ-Linoleic | C18:3n-6c | 0.00 | 0.26 | 0.00 |
| Octadecatetraenoic | C18:4n-3 | 0.00 | 1.39 | 0.22 |
| Eicosadienoic | C20:2n-6 | 0.00 | 0.22 | 0.04 |
| Homo-γ Linolenic | C20:3n-6c | 0.00 | 0.07 | 0.00 |
| Arachidonic | C20:4n-6c | 0.00 | 0.28 | 0.04 |
| Eicosapentaenoic | C20:5n-3c | 0.00 | 4.74 | 0.85 |
| Docosapentaenoic | C22:5n-3 | 0.00 | 0.87 | 0.11 |
| Docosahexaenoic | C22:6n-3c | 0.00 | 5.71 | 0.89 |
| Total n-3 Fats | | 2.76 | 13.40 | 2.20 |
| Total n-6 Fats | | 31.35 | 50.82 | 72.07 |
| n-6/n-3 ratio | | 11.4 to 1 | 3.8 to 1 | 32.8 to 1 |

Brain Collection and Dissection. Immediately following behavioral testing mice were euthanized with an overdose of pentobarbital (100mg/kg), blood and liver sections were collected and the brain were removed and bisected sagittally. Blood (0.2 ml) was drawn directly from the heart prior to saline perfusion (100 ml). The blood mixed with 0.5M EDTA was immediately centrifuged to separate plasma from red blood cells. Both portions were stored and frozen at -80°C for lipid analysis. After saline perfusion a similar portion from each animal's liver was removed and stored in a 1.5 ml tube at -80°C for lipid analyses. For the brain, the left half was stored overnight in a 4% paraformaldehyde solution for immunohistochemistry. The left half was then transferred to a graded series of sucrose solutions at 4°C ending at 30% sucrose for storage until sectioning with a sliding microtome into 25µm coronal sections for Aβ immunocytochemistry and histology as described in a latter section. The right half of each brain was momentarily placed in a cold saline solution, and then dissected into brain stem, cerebellum, posterior cortex, anterior cortex, striatum and hippocampus; each brain region was transferred to a separate pre-labeled 1.5 ml tube and stored at -80°C for neurochemical analysis of protein carbonyls, lipid peroxidation and cytokines as described below.

Extraction of Brain Protein for Sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A 5% sucrose homogenate (wet weight of tissue/ volume) from frozen mouse hippocampus was prepared and extracted as described by Schmidt et al. (2004). Each hippocampal tissue was individually weighed, and then combined with tissue homogenization buffer (THB) at 2 ml per 100 mg of tissue. In addition to the THB, a

protease inhibitor cocktail was also added at 20% of the wet tissue weight to prevent against degradation of the proteins within the tissue samples. On ice, each of the samples were combined with the calculated amounts of THB + inhibitors and fully homogenized. After homogenization, the samples were frozen and stored at -80°C until diethylamine (DEA) and formic acid (FA) extractions.

DEA extraction was used to separate soluble A β from the tissue homogenate. First, 100 μ l of 5% homogenate was mixed with a 0.4% DEA solution (diluted in 100mM NaCl) over ice. This mixture was then transferred into a thick-walled polycarbonate tube and centrifuged at 100,000 x g for 1 hour at 4°C. Following centrifugation, 170 μ l of supernatant was removed and added to a microcentrifuge tube containing 17 μ l of 0.5M Tris Base, pH 6.8 (1 μ l per 10 μ l of supernatant) and vortexed briefly. These samples were then frozen on dry ice and stored at -80°C for later analysis by the ELISA kits.

FA extraction was used to isolate insoluble A β from tissue homogenate. For this extraction 100 μ l of THB was added to the pellet from the initial polycarbonate tube to return the mixture to the original volume. Following the addition of 220 μ l of 95% FA, pellets were sonicated for 1 minute on ice. This mixture was then centrifuged at 100,000 x g for 1 hour at 4°C. After centrifugation, 52.5 μ l of the intermediate phase of the FA extracted mixture was added to 1 ml of FA neutralization solution. Samples were vortexed, immediately frozen on dry ice, and then stored at -80°C for later analysis by the ELISA kits.

A β Brain Deposition. Sandwich ELISA kits for A β ₁₋₄₀ and A β ₁₋₄₂ analyses were purchased from Signet Laboratories. Briefly, the standard curve for A β ₁₋₄₀ was generated with the maximum value at 2000 ng/ml and the minimum value at 0 ng/ml. Each well of the 96-well plate was loaded in duplicate with either standard curve samples or diluted tissue samples. For the formic acid (FA) extracted tissue, the homogenate was diluted with wash/sample diluent (provided) to a ratio of 1:500 of homogenate to diluent; for the diethylamine (DEA) extracted tissue, the homogenate was diluted with wash/sample diluent to a ratio of 1:400. Once loaded into the plate, the standard curve and tissue samples incubated overnight at 4°C. Following this incubation, the plate was washed, diluted primary antibody was added, and the plate was incubated for 2 hours at room temperature. The plate was washed again, and then incubated for 2 hours with secondary antibody-HRP complex. Following another wash, *o*-Phenylenediamine dihydrochloride (OPD) substrate was added, and incubated in a dark room for 45 minutes. Stop solution (3N H₂SO₄) was added to each well and the optical density was read at 490 nm. All of the above steps were repeated to measure for A β ₁₋₄₂, except that the tissue samples were diluted to 1:100 for the DEA extracted samples, while the same 1:500 dilution was used for the FA extracted samples. The standard curve was generated so that the lowest point on the curve was used to correct all of the tissue samples to account for the background interference. The optical densities were then used to calculate wet brain protein concentrations (pmol/g) for either A β ₁₋₄₀ or A β ₁₋₄₂.

Fatty Acid Extraction, Transesterification and Fast Gas Chromatography.

The extraction was performed on frontal cortex according to the method of Folch et al.

(1957). Briefly, individual samples were thawed and homogenized in 20 volumes of chloroform-methanol (2:1, v/v). The homogenate was partitioned with an aqueous salt solution and a small aliquot of the lower phase (total lipid extract) was used for the fatty acid analysis.

Transesterification was performed according to Lepage and Roy (1986). Briefly, an internal standard solution (providing 10 μ g of 23:0 methyl ester) was combined with an aliquot of the lipid extract and a mixture of methanol-hexane (4:1; v/v) into a borosilicate glass tube. Samples were vortexed and placed on ice; subsequently acetyl chloride was added. The tubes were then capped, placed under nitrogen, and transferred to a heating block at 100°C for 10 minutes, the tubes again vortexed and the caps retightened. Following an additional 50 minutes on the heating block, samples were placed on ice, uncapped and neutralized by addition of K₂CO₃. The samples were then centrifuged to remove emulsion and to separate the mixture into two phases. The upper phase was collected and evaporated under a stream of dry nitrogen to a volume of 60 μ l. This solution was transferred to a gas chromatography (GC) vial for fast GC analysis.

A fused silica capillary column (DB-FFAP) of 0.1 mm ID x 15 m length with a film thickness of 0.1 μ m was used for the fast GC analysis. The following temperature program was used: initial, 150°C with a 0.25 minute hold; ramp: 35°C/minute to 200°C, 8°C/minute to 225°C with a 3.2 minute hold, and then 80°C/minute to 245°C with a 2.75 minute hold. The following instrumental conditions were used: carrier gas was H₂ at a flow rate of 56 cm/s and a constant head pressure of 344.7 kPa; FID set at 250°C; air and nitrogen make-up gas flow rates at 450 ml/min and 10 ml/min, respectively; split ratio of 50:1; sampling frequency of 50 Hz; autosampler injections of 2 μ l volume. Individual

fatty acids were identified by comparison with retention times of internal standards and calculated as mean percentage of total frontal cortex fatty acids.

Cytokines. Transgenic AD mice produce pro-inflammatory cytokines that might play a role in the AD-like pathology exhibited by these mice. Therefore, to elucidate the role of fatty acid dietary incorporation on inflammation, pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-2, IL-6, IL-12p70, TNF- α , IFN γ , and GM-CSF) and anti-inflammatory cytokines (IL-4, IL-10) were determined using the RayBiotech Custom Mouse Cytokine Antibody Array within plasma. Briefly, membranes containing the antibodies to the previously mentioned 10 cytokines were blocked against unspecific binding and subsequently incubated for one hour with 1:10 diluted plasma samples from each APP/PS1 and NT mouse. One NT mouse was deleted due to limited space requirements for the kit. The same NT mouse was also deleted from RAWM analysis due to non-performance. The membranes were then incubated with 1x secondary biotinylated antibodies for one hour, followed by incubation with HRP-conjugated streptavidin overnight at 4°C. Between each of the incubations, the membranes were washed with provided washing buffer solutions. Detection of cytokine expression was determined with a two-minute incubation of provided detection buffers and using Fujifilm AR x-ray film that was allowed to develop for 7 seconds. A Kodak DC290 digital camera was used to take back-lit photographs of the AR film. The mean intensity of each signal was determined by densitometry and quantified by the Kodak ID Imaging Analysis Software. Each of the signals were standardized to a zero to one scale based on minimum and maximum mean intensity readings for each cytokine. This was necessary due to the

naturally occurring variability (100x-1000x) in expression levels among the various cytokines. Relative cytokine expression levels among the four groups were compared using the standardized mean signal intensities.

IV. Behavioral Testing Procedures

Open Field. Open field measured exploratory behavior and general activity.

Mice were individually placed into an open black box 81 x 81 cm with 28.5 cm high walls. This area was divided by white lines into 16 squares measuring 20 x 20 cm. Lines crossed were counted for each mouse over a five-minute period.

Balance Beam. This task measured balance and general motor function. The mice were placed on a 1.1 cm wide beam 50.8 cm long suspended 46 cm above a padded surface by two identical columns. Attached at each end of the beam was a 14 x 10.2 cm escape platform. Mice were placed on the beam in a perpendicular orientation and were monitored for a maximum of 60 seconds. The time spent by each mouse on the beam before falling or reaching one of the platforms was recorded for each of 3 successive trials. If a mouse reached one of the escape platforms, a time of 60 seconds was assigned for that trial. The average of all three trials was calculated and recorded.

String Agility. To assess forepaw grip capacity and agility, mice were placed in the center of a taut cotton string suspended 33 cm above a padded surface between the same two columns as in the balance beam task. Mice were allowed to grip the string with only their forepaws and then released for a maximum of 60 seconds. A rating system, ranging between 0 and 5, was employed to assess string agility for a single 60-second trial. A string agility score was given to each mouse based on the following scale: “0”

was awarded for mice that were unable to hang for even a short period of time, “1” if the mouse hung by both forepaws for 60 seconds without escape, “2” was given if the mouse hung with both forepaws plus one hind limb, “3” if the mouse hung with all four paws around the string with no escape, “4” if the mouse had all four paws plus tail around the string, and “5” was given if the mouse escaped to one of the vertical support columns.

Y-maze. To measure general activity and basic memory function, mice were allowed 5 minutes to explore a black Y-maze with 3 arms. Each arm measured 21 x 4 cm with 40 cm high walls. Mice were placed in the center of the maze facing the center area and allowed to explore for 5 minutes, with the number and sequence of arm choices being recorded. General activity was measured as the total number of arm entries, while basic mnemonic function was measured as a percent spontaneous alternation (the ratio of arm choices differing from the previous two choices divided by the total number of entries). For example, the sequence of arm entries (2,3,1,3,2,1,2,3) has six alternation opportunities (total entries minus two) and the percent alternation would be 67%.

Elevated Plus Maze. To measure anxiety/emotionality, mice were placed in the center of an elevated plus maze 82 cm above the floor. The maze consisted of two opposite “open” and two opposite “closed” arms, each 30 x 5 cm; 15 cm high black aluminum walls surrounded the closed arms. The mice were placed in the 5 x 5 cm maze center, facing a closed arm, and were allowed to explore for 5 minutes. The total number of closed arm entries, open arm entries and total time (seconds) spent in the open arms was recorded.

Morris Water Maze. To measure reference learning (acquisition) and memory retention, mice were placed in a 100 cm pool that was divided into 4 equal quadrants by

black lines drawn on the floor of the pool. A transparent 9 cm platform was placed 1.5 cm below the surface of the water in the center of quadrant two. An assortment of visual cues surrounded the pool. For each of four successive one-minute trials per day, mice were started from a different quadrant; the same quadrant start pattern was used across 10 days of acquisition. Latency to find the platform (maximum of 60 seconds) was recorded for each trial and the four daily trials were averaged for statistical analysis. Once a mouse found the platform, it was allowed to stay on it for 30 seconds; if a mouse did not find the platform, it was gently guided to the platform and given the 30 second stay. Animals that did not find the platform were given a latency of 60 seconds. On the day following acquisition testing (day 11), a memory retention (probe) trial was done. For this 60-second trial, the platform was removed and the mouse was started in the quadrant opposite the platform-containing quadrant. Percent time spent in each quadrant, annulus crossings, and average swim speed was determined from videotape recordings of this probe trial.

Circular Platform. As a test of spatial (reference) learning and memory, mice were placed in a 69 cm circular platform with 16 equally-spaced holes on the periphery of the platform. Underneath only one of the 16 holes was a box filled with bedding to allow the mouse escape from aversive stimuli. The aversive stimuli included two 150-watt flood lamps hung 76 cm above the platform and one high-speed fan 15 cm above the platform. Each mouse was given one 5-minute trial per day to explore the area. Between each animal's daily trial, the box position was changed to a different one of three possible escape holes and the platform was cleaned with a dilute vinegar solution to control for olfactory cues. The box position was changed between mice so that different mice had

different escape holes, but any given mouse maintained the same escape hole across all days of testing. Prior to day 1 of testing, two consecutive “shaping” trials were performed wherein mice were placed in the center of the platform and gently guided to their escape location. For the single trial administered on each of 8 test days, mice were placed in the center of the platform facing away from their escape hole and given 5 minutes to explore. Escape latency was measured (maximum of 300 seconds), as was the total number of errors (e.g., the number of head pokes into non-escape holes).

Platform Recognition. This task measured the ability to search for and identify/recognize a variably-placed visible platform. Although this task requires good vision, it is a cognitive-based task because: 1) it necessitates animals to change from the Morris maze’s spatial strategy to a recognition/identification strategy, and 2) it requires animals to ignore the spatial cues present around the pool, which was the same pool used in earlier Morris water maze testing. The platform recognition task employed a 9-cm circular platform raised 0.8 cm above the surface of the water, with a prominent 10 x 40 cm black ensign attached. Mice were started from the same location in the pool for four 60-second maximum trials per day for 4 days. For each trial, the platform location was changed to a different one of the four quadrants. Mice were allowed a maximum of 60 seconds to search/identify and ascend the platform, with a 30-second stay if they located the platform. Mice that did not find the platform within 60 seconds were gently guided to the platform by the experimenter and allowed to stay for 30 seconds. For statistical analysis, escape latencies for all four daily trials were averaged.

Radial Arm Water Maze. Working (short-term) memory was evaluated in the radial arm water maze (RAWM) task, using the same pool that was involved in both

Morris water maze and platform recognition testing. This task also used the same clear platform and visual cues as in Morris maze testing. For RAWM testing, however, an aluminum insert was placed in the pool to create 6 radially distributed swim arms 30.5 cm in length and 19 cm wide emanating from a central circular swim area 40 cm in diameter. The insert extended 5 cm above the surface of the water. The last of four consecutive acquisition trials (T4) and a 30-minute delayed retention trial (T5) were indices of working memory. On any given day of testing, the submerged clear platform was placed at the end of one of the six swim arms. The platform location was changed daily to a different arm in a semi-random pattern. For both studies (I and IIB), mice were tested for 9-12 days. On each day, different start arms for each of the 5 daily trials were selected from the remaining 5 swim arms in a semi-random sequence that involved all 5 arms. For any given trial, the mouse was placed into that trial's start arm facing the center swim area and given 60 seconds to find the platform with a 30 second stay. Each time the mouse entered a non-platform containing arm it was gently pulled back into the start arm and an error was recorded. An error was also recorded if the mouse failed to enter any arm within 20 seconds (in which case it was returned to that trial's start arm) or if a mouse entered the platform-containing arm but did not find the platform. If the mouse did not find the platform within a 60-second trial, it was guided by the experimenter to the platform, allowed to stay for 30 seconds, and was assigned a latency of 60 seconds. An error was assigned to any animal that, for any one minute trial, did not find the goal arm and refused to make at least 3 choices on their own during that trial. This number was calculated by averaging errors for all animals that did not locate the

platform for Block 1 (day 1 through day 3) on trial 1. Both the number of errors (incorrect arm choices) and escape latency were recorded for each daily trial.

V. Brain A β Deposition Determinations

A β Immunostaining. The “total A β ” primary antiserum was raised against A β_{40} in rabbits and recognized A β_{40} and A β_{42} via ELISA assays. Antiserum selective for both A β_{1-40} and A β_{1-42} was purchased from Quality Controlled Biochemical (Hopkinton, MA). Confirmation of selectivity was provided by preabsorption experiments, which blocked all punctuate staining. Brain sections were incubated with the primary antibody overnight at 4°C. The sections were then incubated in biotinylated secondary antibody for two hours followed by streptavidin-peroxidase. The peroxidase reaction consisted of a solution of 1.4mM diaminobenzidine and 0.03% hydrogen peroxide in PBS for five minutes.

Congo Red Staining. Congo red staining for compact A β deposition was performed on slide-mounted sections that were dried for at least 12 hours and then rehydrated for approximately 30 seconds prior to staining. The hydrated slides were incubated in a freshly prepared alkaline alcoholic saturated chloride solution consisting of 2.5mM NaOH in 80% reagent alcohol (95% ethanol and 5% isopropanol) for 20 minutes. The sections were then incubated in 0.2% Congo red in a freshly prepared and filtered alkaline alcoholic saturated sodium chloride for 30 minutes. The slides were rinsed with three rapid changes of 100% ethanol, cleared through three rapid changes of xylene, and finally cover slipped with Permount.

Quantification of A β deposition. The Oncor V150 image analysis system was used to quantify stained brain sections. This software used hue, saturation and intensity (HIS) to segment objects within the image field. Standard slides were used, which contain extremes of staining intensities, to establish thresholds for object segmentation. The operator for image analysis remained blind with respect to genotype or behavioral assessment. For each animal, frontal cortex and hippocampus was quantified from four horizontal sections spaced 600 μ m apart beginning at 2000 μ m ventral to bregma. The frontal cortex measurement used an 80x field with one limit as the edge of the cortex and the other limit as the midline in the most anterior position possible. The total cortical measurement area consisted of a rectangular 850,000 μ m². This area was primarily comprised of the middle two-thirds of the cortical mantle (e.g., cortical layers 1 and 6 are excluded). The hippocampal analyses involved three sub-areas: CA1, CA3, and dentate gyrus. The areas measured were comprised of 6-8 horizontal sections equally spaced within each region of CA1, CA3 and dentate gyrus. Region CA1 was defined as the pyramidal cells on the opposite side of the hippocampal fissure from the dentate gyrus. The pyramidal cell region adjacent to the dentate gyrus, not including the granule cell layers, encompassed region CA3. The objective was positioned so that the hilus of the dentate gyrus was in the center for analysis of this region. “A β load” referred to the percent area within the measurement field occupied by the reaction product, while “Congo red staining” referred to the percent area stained with Congo red. All values for a given mouse and given brain area were averaged to represent a single value for that animal that was used in the statistical analysis.

VI. Statistical Analysis

A total of 19 behavioral measures were obtained from the 9-behavioral task battery previously described. The tasks were divided into single day tasks and tasks that involved multiple test days. All of the single day tasks (open field, balance beam, Y-maze and elevated plus maze) except string agility were analyzed using one-way ANOVA. String agility was analyzed using the Kruskal-Wallis non-parametric test and *post hoc* Mann-Whitney U test. For multi-day tasks (Morris water maze, circular platform, platform recognition and radial arm water maze) both one-way ANOVAs and two-way repeated measure ANOVAs were employed. Prior to statistical analysis of multi-day measures, data was grouped into 2-5 day blocks (except for platform recognition) to facilitate analysis and presentation. Following ANOVA analysis, *post-hoc* pair-by-pair differences between groups (planned comparisons) was resolved using the Fisher LSD test. Swim speed from the Morris water maze retention trial was calculated using the *Mouse Clocker* software and analyzed by one-way ANOVA; annulus crossings during this trial were analyzed by Mann-Whitney U-tests. All group comparisons were considered significance at $p < 0.05$. Any non-performers (e.g. repeated circulars, consistent floaters, etc.) in a particular task were eliminated from statistical analysis in that task.

To group behavioral and/or pathologic measures based on their common factors, Factor Analysis (FA) was performed using *Systat* software. FA used all collected data, regardless of genotype or treatment, to relate measures into individual factors. Each factor measured a different component of behavior or cognition (i.e. reference memory, sensorimotor function, etc.). In this way, behavioral measures related to one another

could be determined, as well as how performance in one task might be predictive of performance in another task (see Leighty et al., 2004 for more detailed explanation of FA).

For Study I (Life-long Vaccination Study), an FA was performed using all 19 behavioral measures collected at both the 4½-6 month and 15-16½ month time points. An additional DFA was carried out at the 15-16½ month time point that included 8 A β pathologic measures in addition to the 19 behavioral measures. The 8 pathology measures included 4 measures for total A β burden (diffuse + compact) within 3 hippocampal areas (CA1, CA3 & dentate gyrus) and frontal cortex (CX), and 4 measures for Congo red staining (compact A β only) within the same brain areas.

To determine if the three experimental groups (NT, Tg+/Con and Tg+/A β) were distinguishable behaviorally from one another, discriminant function analysis (DFA) was performed using all 19 behavioral measures for both the 4½-6 month and 15-16½ month time points. For the 4½-6 month time point, DFA was carried out with only the 7 measures (all cognitive-based) that loaded within Factor 1. Similarly, the 15-16½ month time point DFA was also performed with only the 9 measures (all cognitive-based) that loaded within Factor 1. All DFA analyses were performed using the *Systat* software with two different DFA methods — direct entry and stepwise-forward. The direct entry method used all behavior measures available, while the stepwise-forward method selected measures based on their variance contribution to best discriminate between the three groups (see Leighty et al., 2004 for more detailed information on DFA methodology).

For Study II, Part B (Omega-3 Behavioral Study), statistical analysis of behavior was similar to Study I, as just described. In addition, separate behavioral analyses including only APP/PS1 and NT mice on either the standard or high n-3 fatty acid diets were evaluated and included after the initial Omega-3 Fatty Acid Study – Part BI section and entitled Omega-3 Fatty Acid Study – Part BII.

For the Omega-3 Fatty Acid Study II neurochemical and histological analyses, groups were compared using one-way ANOVAs, with post-hoc Fisher's test to determine significance and included only the NT and APP/PS1 mice on any of the three experimental diets.

Results

I. Life-long Vaccination Study

Significant differences of transgenic and A β immunotherapy effects are summarized in Table 4.

Table 4. A summary of transgenic and immunotherapy at both 4½-6 and 15-16½ month test points.

| Task | 4½-6 Month | | 15-16½ Month | |
|-------------------------------|----------------------------------|----------------------------|----------------------------------|----------------------------|
| | Transgenic Effect | Immunotherapy Effect | Transgenic Effect | Immunotherapy Effect |
| Open Field Activity | ↑ Tg+/Con, Tg+/A β | _____ | _____ | _____ |
| Balance Beam | _____ | Tg+/A β impaired | _____ | _____ |
| String Agility | _____ | _____ | _____ | _____ |
| Elevated Plus Maze | | | | |
| % open arms | _____ | _____ | ↑ Tg+/Con | ↓ Tg+/A β |
| Open arm entries | _____ | ↑ Tg+/A β | _____ | _____ |
| Closed arm entries | _____ | ↑ Tg+/A β | _____ | _____ |
| Y-Maze | | | | |
| Arm entries | _____ | ↑ Tg+/A β | _____ | ↑ Tg+/A β |
| % Alternation | _____ | _____ | _____ | _____ |
| Morris Maze Acquisition | Tg+/Con impaired | Tg+/A β not impaired | Tg+/Con impaired | Tg+/A β not impaired |
| Morris Maze Retention | Tg+/Con impaired | Tg+/A β not impaired | Tg+/Con & Tg+/A β impaired | _____ |
| Circular Platform Acquisition | _____ | _____ | _____ | _____ |
| Platform Recognition | _____ | _____ | Tg+/Con impaired | Tg+/A β not impaired |
| Radial Arm Water Maze | | | | |
| Overall | Tg+/Con & Tg+/A β impaired | _____ | Tg+/Con impaired | Tg+/A β not impaired |
| Last Block | Tg+/Con impaired | Tg+/A β not impaired | Tg+/Con impaired | Tg+/A β not impaired |

Abbreviations: Tg+/Con = transgenic control mice; Tg+/A β = transgenic A β vaccinated mice; ↑ = increased; ↓ = decreased.

Sensorimotor- and Anxiety-based Tasks.

Open Field and Y-maze Entries. At 4½-6 months of age, both Tg+/Con and Tg+/Aβ exhibited increased open field activity (Fig. 6a) compared to NT control mice ($P < 0.02$). However, at the later time point, 15-16½ months of age, there were no differences between the groups. Within the Y-maze task for activity/exploration, arm entries of the Tg+/Aβ group were elevated at both early ($P < 0.01$) and late ($P < 0.05$) test points compared to both Tg+/Con and NT groups (Fig. 8a).

Balance Beam. In the balance beam task (Fig. 6b), 4½-6 month old Tg+/Aβ exhibited poorer performance than the NT mice; the Tg+/Con group showed an intermediate balance ability. However, at 15-16½ months, all three groups exhibited poor balance ability as indicated by short trial times prior to falling from the beam.

String Agility. Despite differences in activity (from open field or Y-maze entries) and balance performance, there were no differences at either test point in string agility (Fig. 6c). This shows that any age- and genotype/treatment-related deficits exhibited in sensorimotor tasks are task-specific and did not deleteriously affect performance in cognitive-based tasks.

Elevated Plus-Maze. In the elevated plus-maze task for anxiety and/or emotionality at 4½-6 months of age, an increased number of both open and closed arms entries was exhibited by Tg+/Aβ mice, but not the Tg+/Con mice, compared with NT control mice (Fig. 7b and c). This is more reflective of an increase in activity level (in open field and Y-maze entries) and less of a decrease in anxiety. This is especially evident since arm entries in elevated plus-maze generally load with other activity measures in Factor Analysis (see below). Despite the increased number of entries, there

were no differences seen between all three groups in the percent time spent in the open arms at the younger test age (Fig. 7a). However, at the aged test point, Tg+/Con mice exhibited less anxiety by spending an increased percent time in the open arms compared to NT controls ($P < 0.02$). Though there were no group differences in the number of entries into either closed or open arms at this age (Fig. 7b and c).

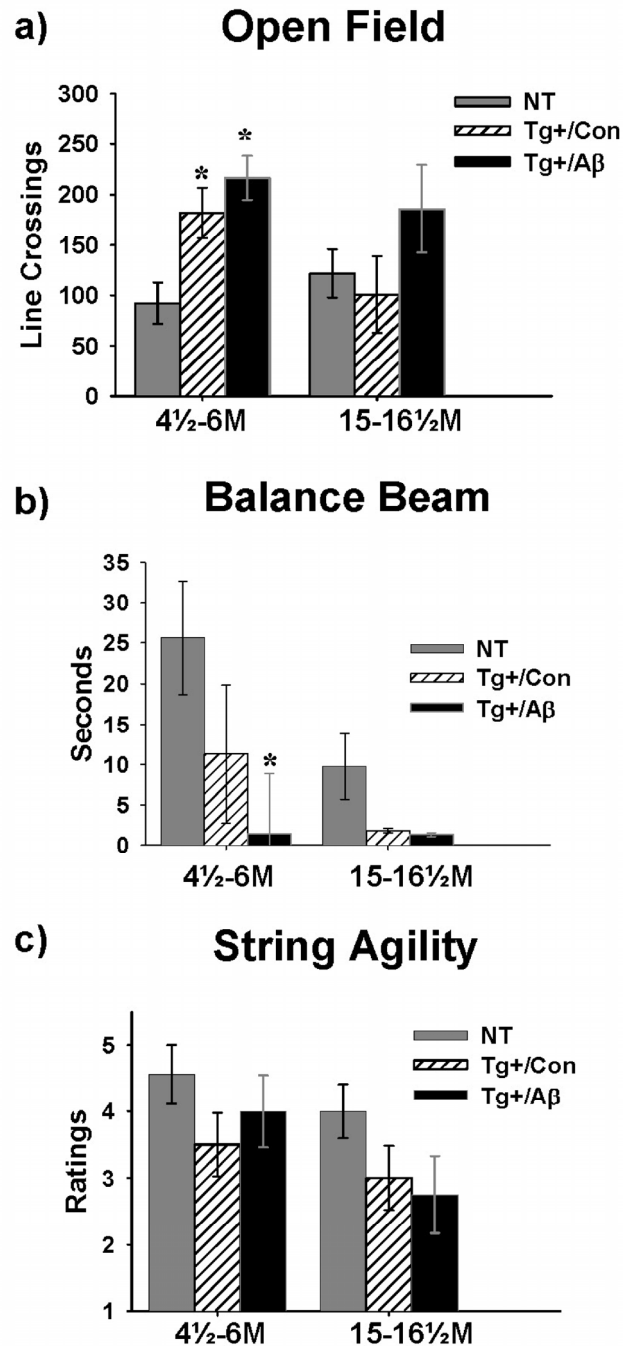


Fig. 6. Comparison of sensorimotor function in NT, Tg+/Con, and Tg+/Aβ mice at 4½ - 6 and 15-16½ behavioral test points. Data represent the mean ± S.E.M. (a) Open field activity, determined by open field line crossings, was increased in young adult Tg+/Con and Tg+/Aβ mice (b) Equilibrium/agility, as measured by time on a balance beam, was impaired in young Tg+/Aβ mice. (c) String agility, as measured by forepaw grip suspension. *Significantly different from NT at the age indicated, with $P < 0.05$ or higher level of significance.

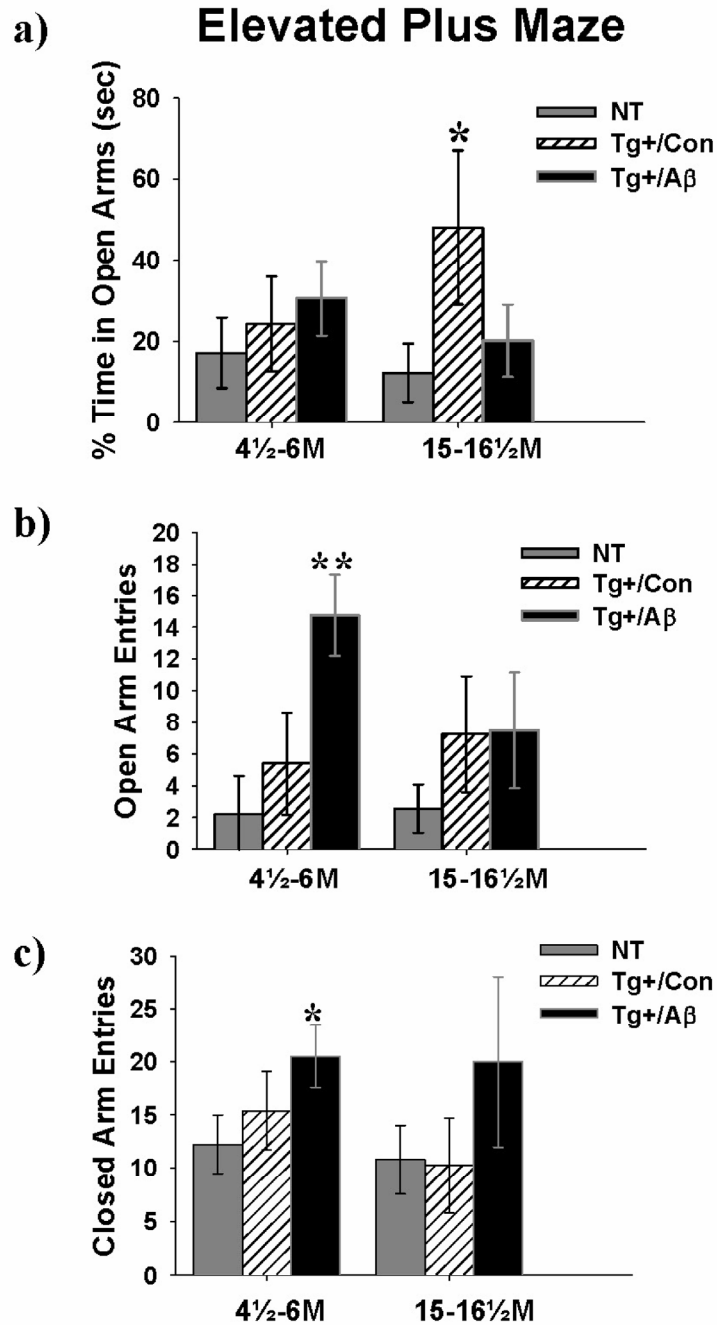


Fig. 7. Anxiety/emotionality, as determined in the elevated plus-maze by percent time in open arms and arm entries. NT, Tg+/Con, and Tg+/A β mice were evaluated at adult (4½-6 month) and aged (15-16½ month) test points. (a) Aged Tg+/Con mice spent an increased percent time in open arms compared with NT controls. (b) The number of open arm entries was elevated in adult Tg+/A β mice in comparison to both NT and Tg+/Con mice. (c) Adult Tg+/A β mice also had an increased number of closed arm entries. Data represent the means \pm S.E.M. *Significantly different from NT mice at $P < 0.05$, **significantly different from adult NT and Tg+/Con mice at $P < 0.05$.

Cognitive-based Tasks

Y-maze Alternation. There was no transgenic effect at either test point for percent alternation within the Y-maze task (Fig. 8b), thus no protective effect of A β vaccination could be observed for this task.

Morris Water Maze Acquisition. To facilitate presentation and statistical analysis, the Morris maze acquisition data was evaluated as two 5-day blocks (Fig. 9). At the adult test point, there was an overall groups effect across both blocks [$F(2,19)=7.37$; $P<0.005$], with the Tg+/Con exhibiting impairment compared to the two remaining groups. Also across both blocks, the Tg+/Con were found to have significantly higher escape latency, compared to both NT ($P<0.005$) and Tg+/A β ($P<0.02$) mice; the latter two groups did not differ in their acquisition performance. At the aged test point, 15-16½ months, there was nearly an overall groups effect [$F(2,15)=3.28$; $P=0.07$]. Planned comparisons revealed a significant impairment across both blocks for Tg+/Con mice compared with NT controls ($P<0.05$). In contrast, the performance of Tg+/A β mice was no different from that of NT control mice. Additionally, across both blocks at both the young and aged test point, the Tg+/A β were no different from the NT controls. Overall, A β immunotherapy protected APP/PS1 transgenic mice against memory impairment that was present in control Tg+ mice at an adult age and this protection was preserved into old age.

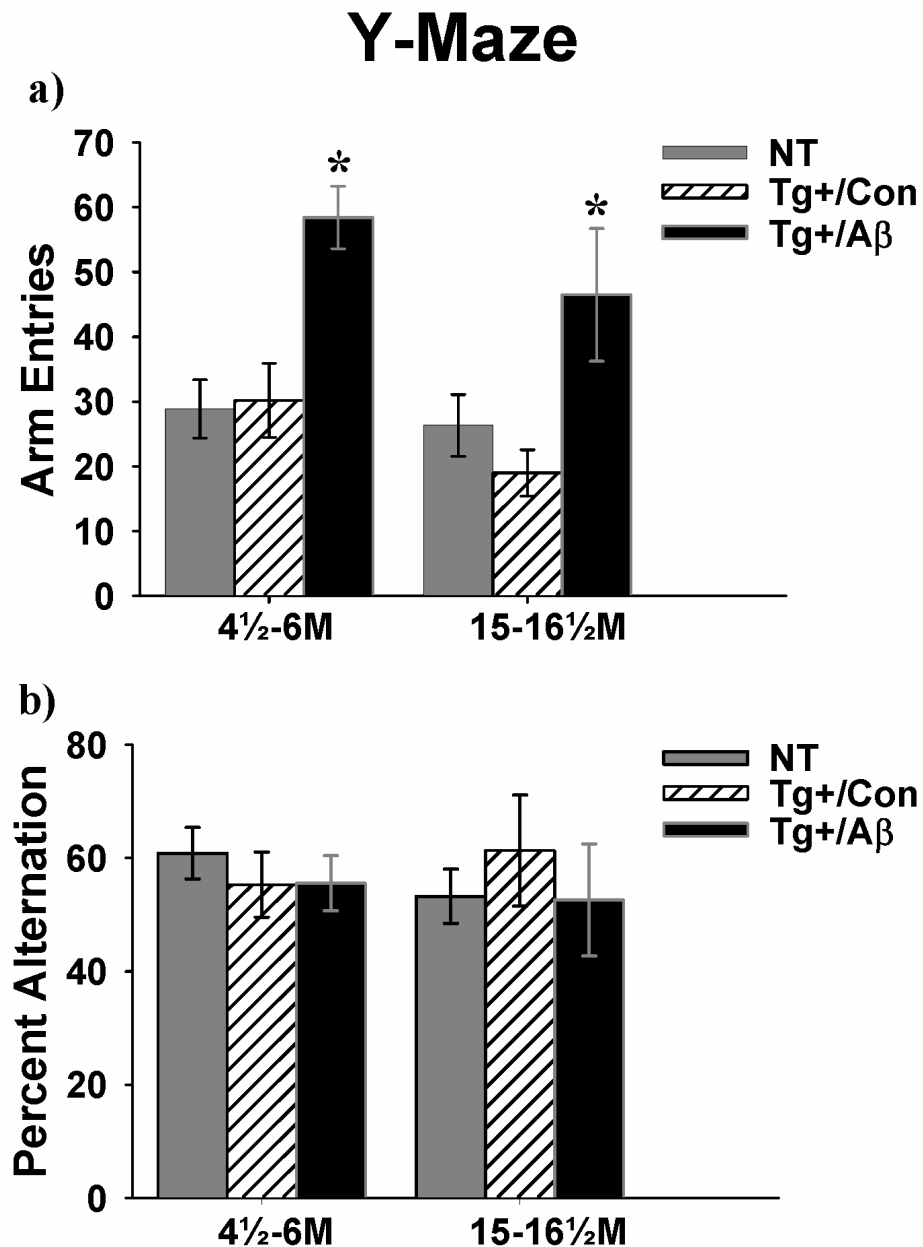


Fig. 8. Y-maze arm entries (a) and percent spontaneous alternation (b) for adult and aged NT, Tg+/Con, and Tg+/A β mice. Data represent the mean \pm S.E.M. (a) Tg+/A β mice had significantly more arm entries (e.g. increased activity) compared with both NT and Tg+/Con mice at the 4½ -6 month adult test point. At the 15-16½ month aged test point, Tg+/A β mice continued to exhibit an increased number of arm entries compared with NT controls. (b) There were no group differences at either age for percent spontaneous alternation. *Significantly different from NT and Tg+/Con at $P < 0.05$, or higher level of significance.

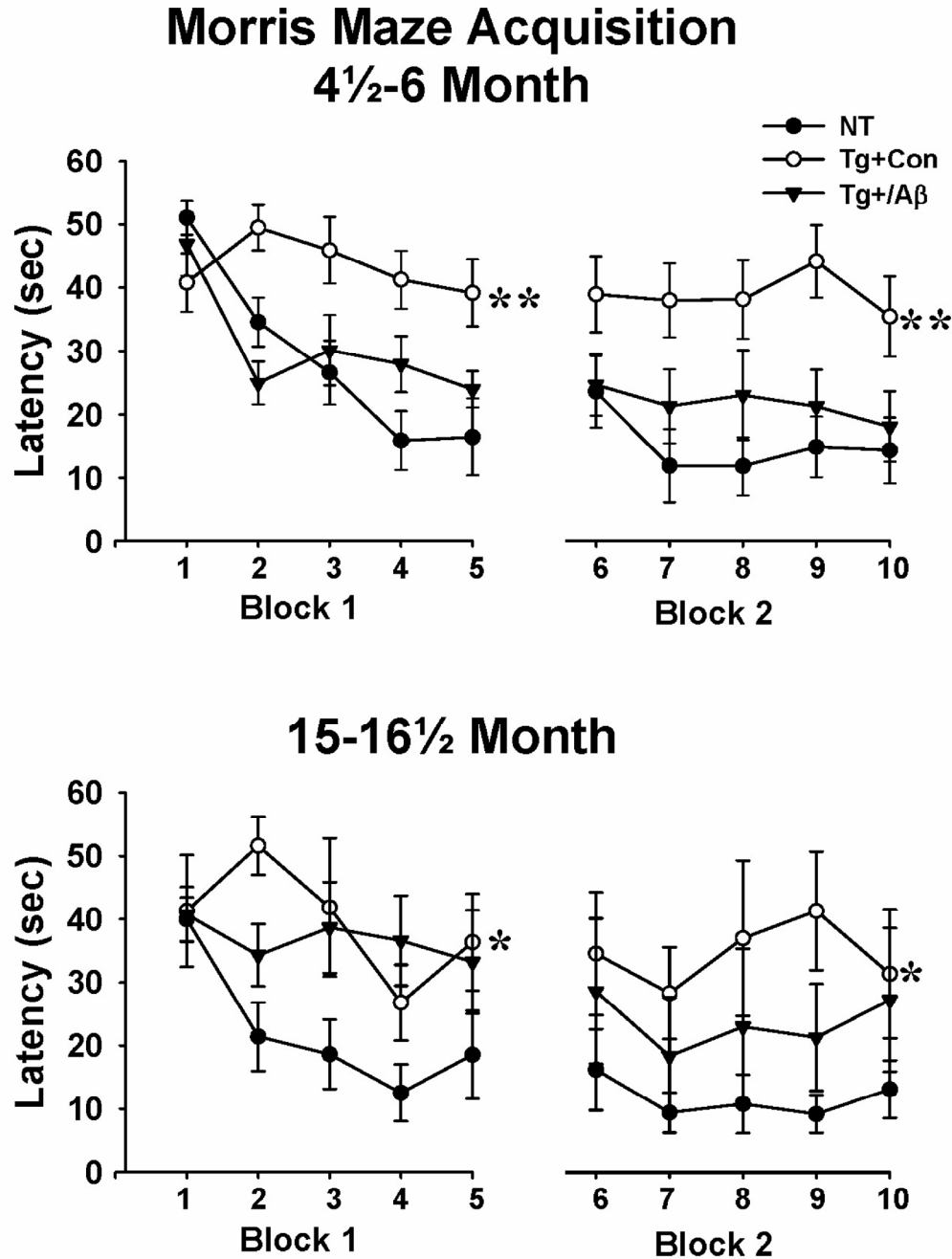


Fig. 9. Morris water maze acquisition at adult (4½-6 months) and aged (15-16½ months) time points for NT, Tg+/Con, and Tg+/Aβ mice. The 10 days of acquisition, as measured by latency to find a submerged stationary platform, are presented in two 5-day blocks. For both blocks and at both test points, Tg+/Con mice were significantly impaired, while performance of Tg+/Aβ mice was no different than NT mice. **Significantly different from both other groups at $P < 0.02$ or higher level of significance, *significantly different from NT group at $P < 0.05$.

Morris Water Maze Retention. The Morris water maze probe trial is presented below in Fig. 10 for both adult (4½-6 months) and aged (15-16½ months) test points. Both adult NT and Tg+/Aβ groups showed an exclusive preference for the quadrant that formerly contained the submerged platform (Q2). In contrast, the adult Tg+/Con mice showed no quadrant preference as seen by their having similar percent time spent in each quadrant. Also, the Tg+/Con mice had significantly fewer annulus crossings compared to the NT mice ($P<0.05$), while the Tg+/Aβ mice had similar annulus crossings to the NT group. This shows that at the adult test point, Aβ immunotherapy protects against cognitive impairment of memory retention that was present in Tg+/Con mice. At the aged test point, similar to the adult mice, the NT group showed an exclusive quadrant preference for the former platform-containing quadrant (Q2). However, neither group of Tg+ mice showed such a preference. In addition, at the aged test point, there were no group differences in annulus crossings; neither were there any group differences in swim speed at either test point exhibited during this probe trial.

Morris Maze Retention

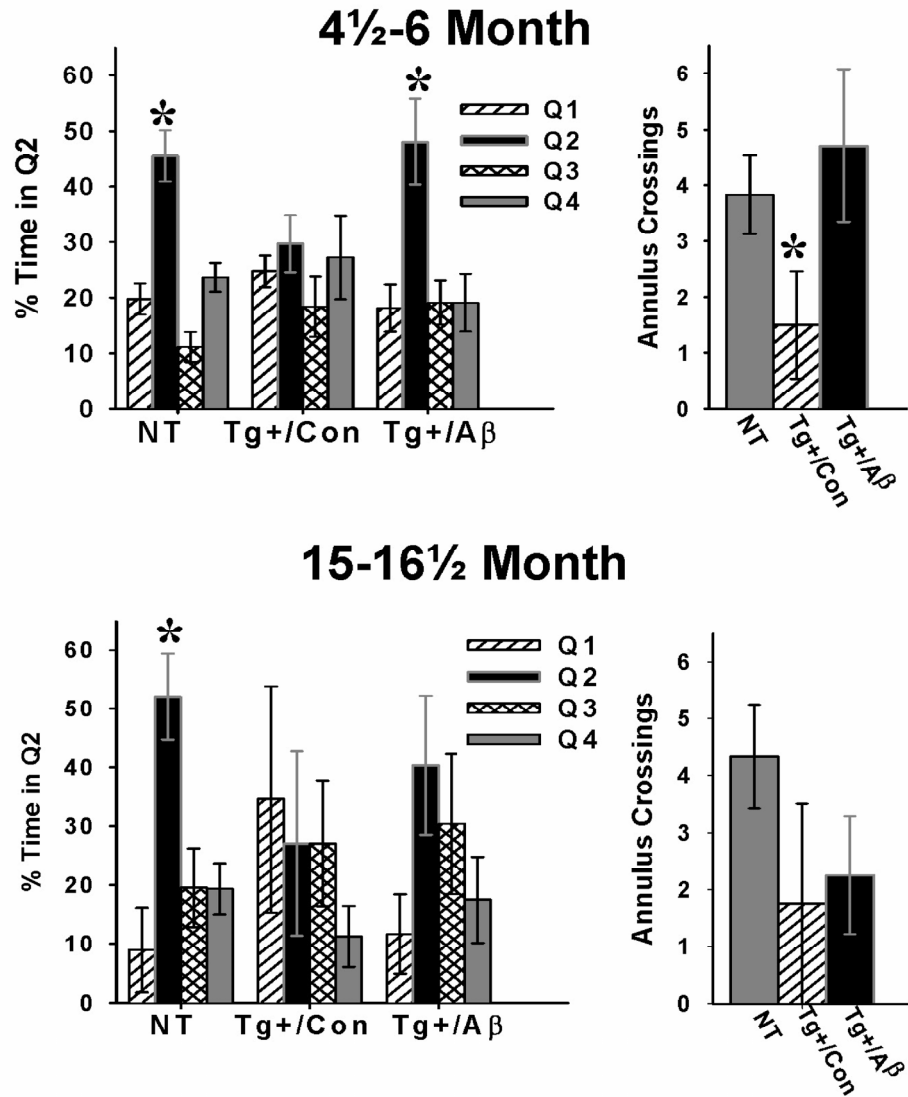


Fig. 10. Probe trial testing for reference memory retention, assessed on the day following the completion of water maze acquisition. A single 1-minute trial was done, with the percent of time spent in the quadrant formerly containing the submerged platform (Q2) and the number of annulus crossings determined. At the 4½-6 month test point, NT and Tg+/Aβ mice showed an exclusive quadrant preference for Q2 compared with all other quadrants, while Tg+/Con mice exhibited no quadrant preference. *Significantly higher than all other quadrants at $P < 0.0001$ (NT group) or $P < 0.01$ (Tg+/Aβ group). Tg+/Con mice also had significantly fewer annulus crossings compared with NT mice ($P < 0.05$), while there was no difference between the other two groups. At 15-16½ months of age, only NT mice showed a quadrant preference for Q2 (*significantly higher than all other quadrants at $P < 0.0001$). There were no group differences in annulus crossings at this age.

Circular Platform. There were no impairments in latency to find the escape hole (Fig. 11a and b) or number of head pokes/errors into non-escape holes (Fig. 12a and b) for Tg+/Con mice at either test point in the circular platform task for spatial learning/memory. Therefore, no protective effect of A β immunotherapy could be observed in this task. In addition there were no differences at either the adult or aged test points between any of the groups; however, strong overall effects of learning across days were present at both adult [$F(7, 133)=10.75$; $P<0.0001$] and aged [$F(7,112)=7.24$; $P<0.0001$] test point in circular platform latency.

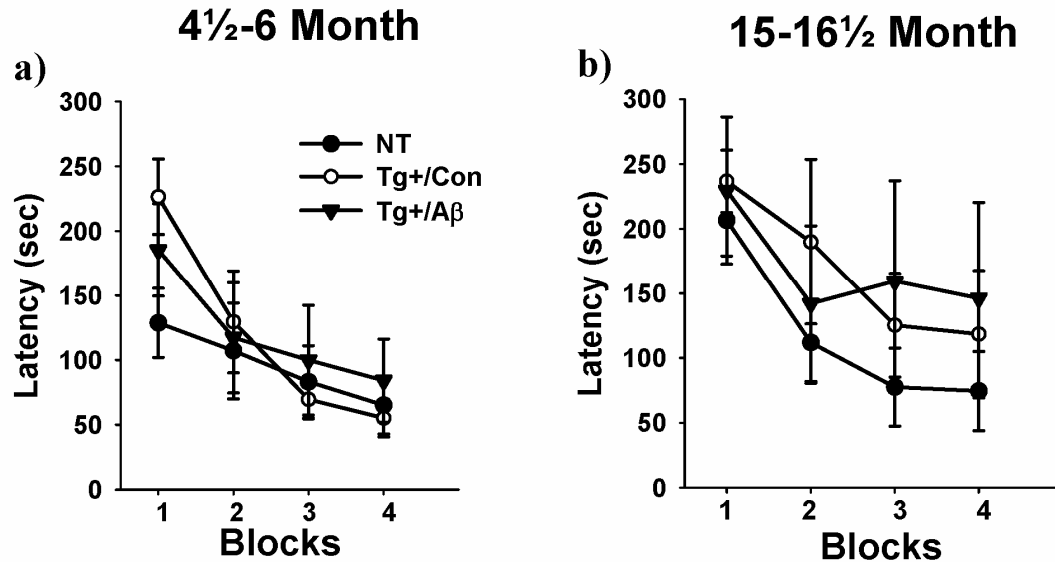


Fig. 11. Spatial learning/memory, as determined by the circular platform task latency to find the escape hole across 4 blocks consisting of 2 days each. NT, Tg+/Con, and Tg+/A β were behaviorally evaluated at both adult (4½-6 months) and aged (15-16½ month) test points. There were no group differences at either adult (a) or aged (b) test points. However, there was a strong overall effect of learning across days at both adult and aged test points.

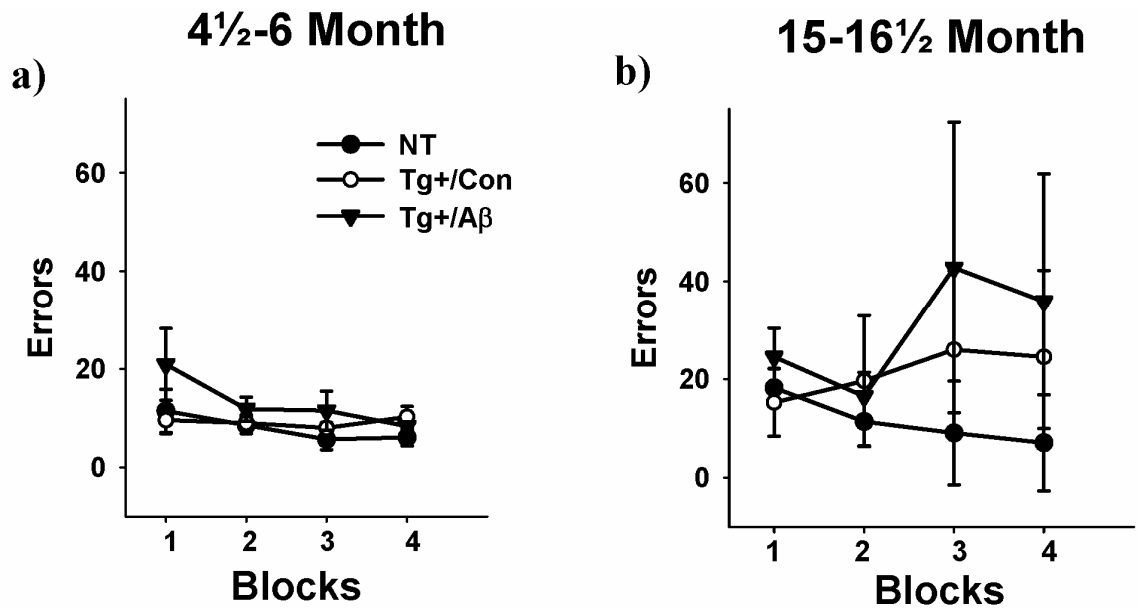


Fig. 12. Spatial learning/memory, as determined by the circular platform task the number of errors, indexed as the number of head pokes into holes which did not contain the escape hole, across four 2-day blocks. NT, Tg+/Con, and Tg+/Aβ were behaviorally evaluated at both adult (4½-6 months) and aged (15-16½ month) test points. There were no group differences at either adult (a) or aged (b) test points.

Platform Recognition. At the 4½-6 month test point, all groups collectively exhibited a strong learning effect across days [$F(3, 54)=20.91$; $P<0.0001$], and there were no overall group differences across the 4 days of testing (Fig. 13). Contrary, there was an overall groups effect over all 4 days of testing at the 15-16½ month time point [$F(2,15)=3.97$; $P<0.05$]. At this age, the Tg+/Con mice were impaired overall versus NT controls ($P<0.02$) and specifically on days 1 and 3 compared to the NT mice. In contrast to the impaired performance of the Tg+/Con mice, the aged Tg+/A β mice performed similar to NT controls overall and for each of the 4 test days (Fig. 13). However, by the last day of testing, there were no group differences in escape latency. This indicates that the Tg+/Con were eventually able to reduce their latencies comparable to the remaining two groups. In a comparison across both test points, the NT and Tg+/A β mice showed similar latencies indicating that their performance did not decline with increased age. In contrast, the escape latency of the Tg+/Con mice was significantly higher at the aged test point compared to the adult test point ($P<0.05$). This indicates that A β immunotherapy protected Tg+ mice from an age-dependent impairment in switching from the spatial strategy of Morris maze to the recognition/ identification strategy of platform recognition.

Platform Recognition

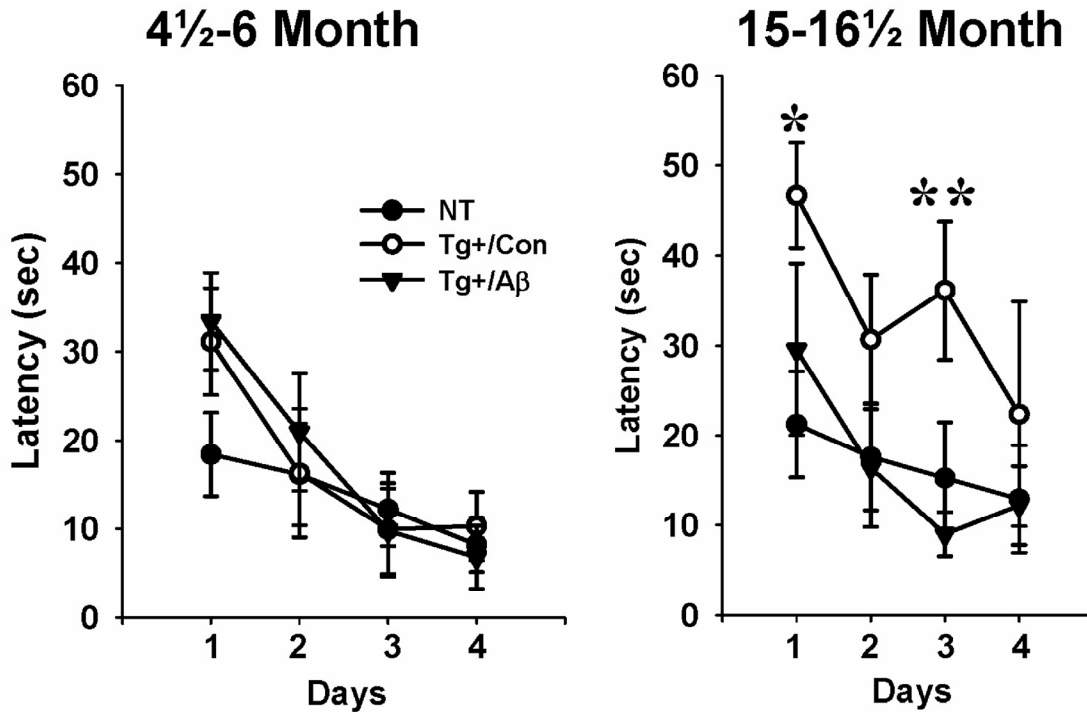


Fig. 13. Platform recognition testing for the ability to search/ identify a variably-placed and conspicuously marked platform over 4 days of testing, with latency to swim to the platform being measured. At the 4½-6 month test point, there were no differences between NT, Tg+/Con, and Tg+/Aβ mice to find the variably placed platform, with all groups reducing their escape latency over days. At the 15-16½ month test point, however, aged Tg+/Con mice were impaired overall, as well as during days 1 and 3 of testing. By contrast, Tg+/Aβ mice performed identically to NT mice. *Significantly different from NT mice at $P<0.005$, **significantly different from both NT and Tg+/Aβ mice at $P<0.025$ or higher level of significance.

Radial Arm Water Maze. The number of errors at the 4½-6 month test point in the Radial Arm Water Maze (RAWM) task is shown in Fig. 14 below. At this age, the data were collected across three 3-day blocks for T1(randomized initial trial), T4 (final acquisition trial), and T5 (delayed retention trial). For block 1 (B1), both groups of Tg+ mice showed memory impairment in the final acquisition trial in addition to the delayed retention trial compared to the NT group, which was effective in lowering their errors by T4 and T5. In contrast, by block 2, the Tg+/A β mice had similar performance to the NT mice on T5, whereas the Tg+/Con mice remained impaired compared to the NT controls. By block 3, the Tg+/A β group performed similar to the NT control mice on both T4 and T5 trials of working memory; in sharp contrast the Tg+/Con exhibited marked memory impairment on both T4 and T5 compared to the NT mice. Over all three blocks of testing, there was a significant effect of groups for both T4 [$F(2,18)=11.30$; $P<0.001$] and T5 [$F(2,18)=9.28$; $P<0.005$] in that both Tg+ groups were impaired compared to the NT controls overall for both T4 and T5 ($P<0.02$ or higher level of significance). This overall impairment of Tg+/A β mice reflects the fact that it required them several blocks of testing to reduce their T4 and T5 errors to the level of the NT controls. Fig. 15 below illustrates the latency in seconds across three 3-day blocks of RAWM behavioral testing at the adult test point. Initially within B1, Tg+/A β mice had significantly higher latencies on T4 and T5 compared to the NT mice. However, by the remaining blocks, they had similar latencies/working memory to the NT control group. Although also not statistically different from NT controls or Tg+/A β mice, Tg+/Con mice exhibited consistently higher latencies than those two groups for T4 and T5 within the last two blocks of testing.

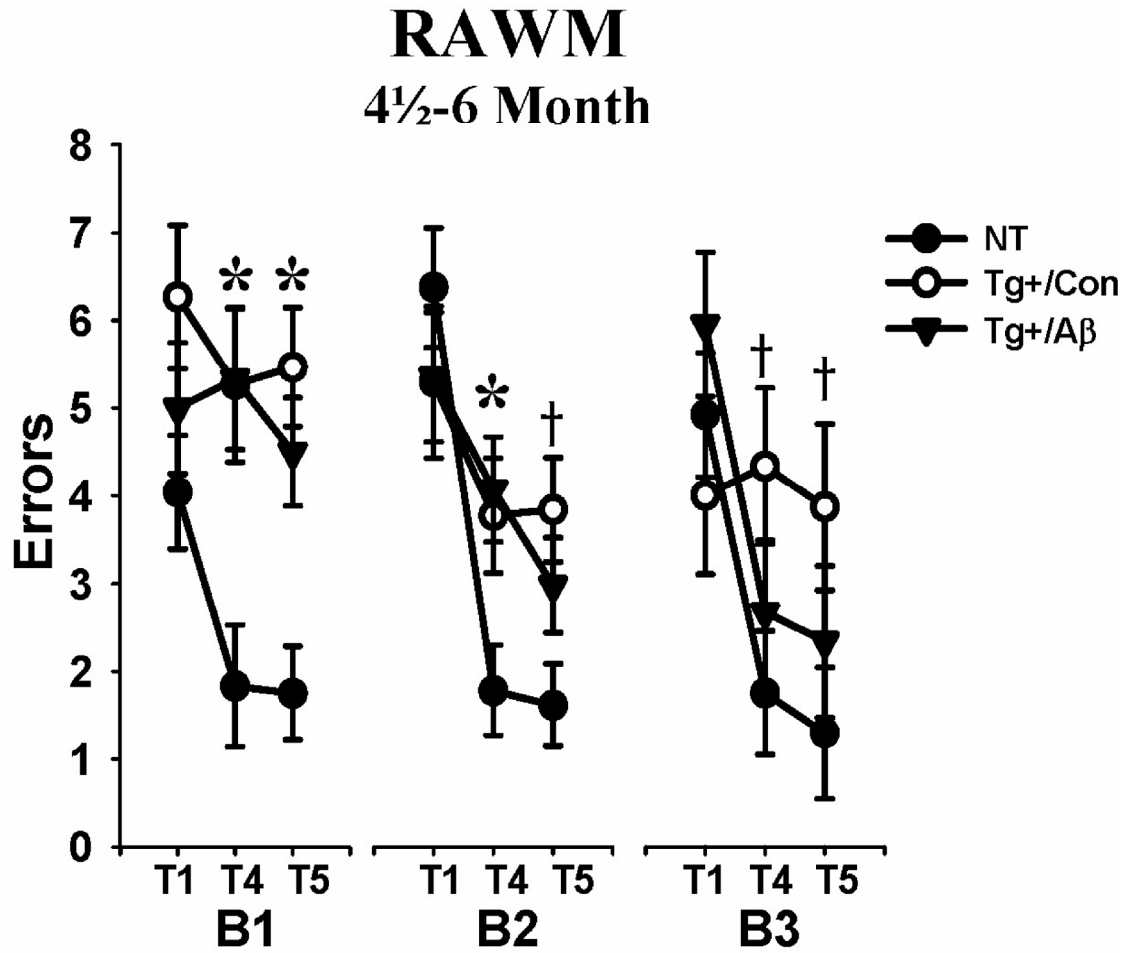


Fig. 14. Working memory in the RAWM task at the 4½-6 month test point, with errors being evaluated for T1, T4, and T5 over three 3-day blocks. T4 and T5 are indices of working memory. By the last block of testing, Tg+/Aβ mice were no different than NT mice in working memory performance, while Tg+/Con mice consistently performed poorly over all three blocks in being unable to reduce their number of errors by T4 and T5 in any block. All data are means (\pm S.E.M.). *NT group significantly better than both Tg+/Con and Tg+/Aβ groups at $P < 0.05$ or higher level of significance, †NT group significantly better than Tg+/Con group at $P < 0.05$ or higher level of significance.

RAWM 4½-6 Month

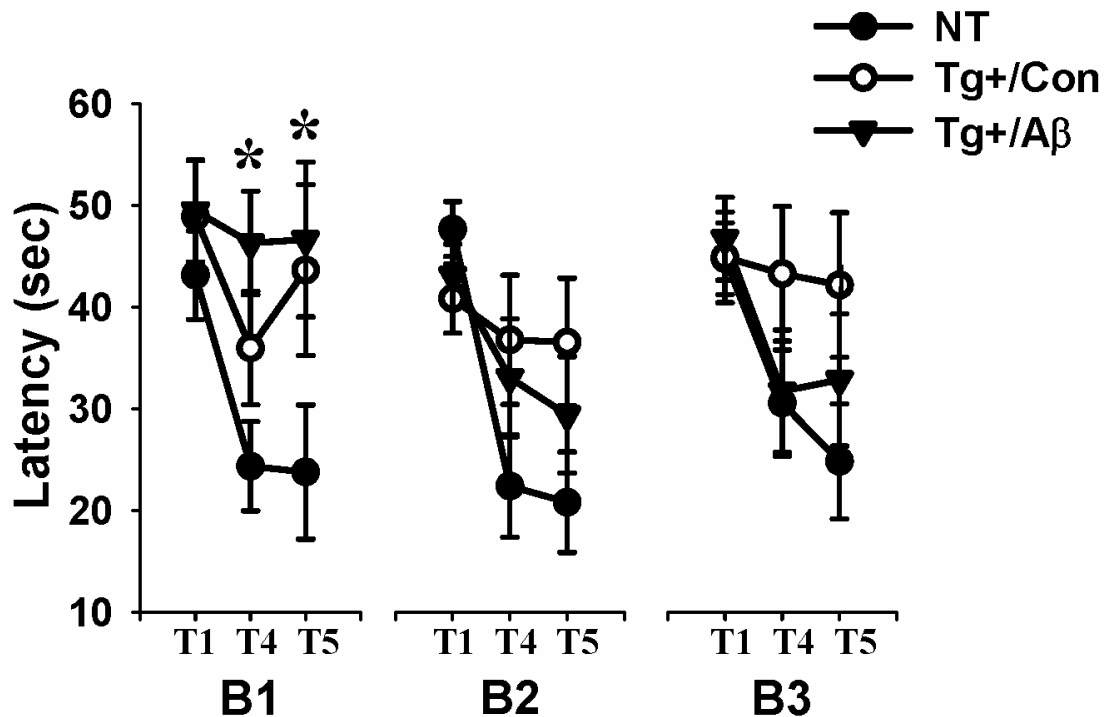


Fig. 15. Working memory in the RAWM task at the 4½-6 month test point, with latency to find the hidden platform being evaluated for T1, T4, and T5 over three 3-day blocks. Within the first block of testing (B1), the NT mice performed significantly better than the Tg+/Aβ mice on T4 and T5, with the Tg+/Con mice having intermediate performance. However, by the last two blocks of testing, Tg+/Aβ mice were no different than NT mice in working memory performance. Although not statistically different from NT controls, Tg+/Con mice consistently had higher latencies and were unable to reduce their latencies by T4 and T5 in any block. All data are means (\pm S.E.M.). *NT group significantly better than Tg+/Aβ group at $P < 0.05$ or higher level of significance.

The number of errors at the 15-16½ month test point in the RAWM task is shown in Fig. 16 below using errors for T1, T4, and T5. Comparisons between the two test points for RAWM can not be made because prior to the aged test point, the walls at the end of each swim arm of the RAWM aluminum insert were painted black. For the first three blocks of behavioral testing, all groups performed similarly with the NT controls having consistently fewer errors than the two Tg+ groups. By block 4, however, the Tg+/Con mice exhibited significantly more errors compared to the NT controls for T4 ($P<0.01$) and T5 ($P<0.01$), while the Tg+/A β mice reduced their errors similar to that of the NT mice. In addition, Tg+/Con mice were significantly impaired by having a higher number of errors in overall T5 performance ($P<0.05$), in contrast to the performance of Tg+/A β mice which was identical to that of NT mice. The protective effect of A β immunotherapy to Tg+ mice is underscored by RAWM escape latency (Fig. 17), particularly during the last block. During trial 4 of block 4, Tg+/A β mice performed identical to NT mice, while Tg+/Con were impaired compared to both Tg+/A β and NT mice. Likewise, during T5, the Tg+/A β mice had similar latencies to the NT mice, while the Tg+/Con mice were significantly impaired compared to the NT mice. In addition, Tg+/Con mice were impaired by having substantially higher latencies in overall T4 ($P<0.02$) and overall T5 ($P<0.02$) across all four blocks in contrast to Tg+/A β which performed similar to NT mice in those trials.

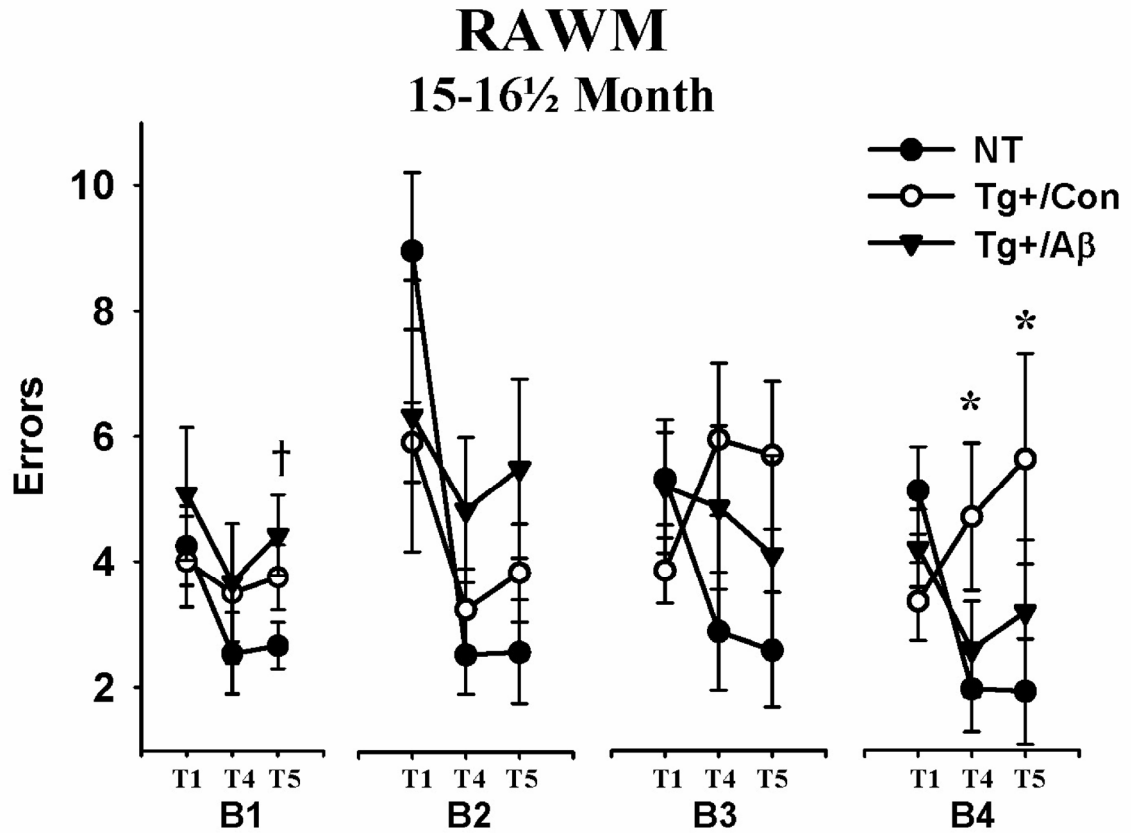


Fig. 16. RAWM testing for working memory at the 15-16½ month test point, with number of errors indicated for T1, T4, and T5 over four 3-day blocks of testing. For the first three blocks of testing, all groups performed similarly. By the final block, however, Tg+/Aβ mice reduced their T4 and T5 errors down to the level of NT controls, while Tg+/Con mice made significantly higher numbers of errors compared with NT mice. †Tg+/Aβ group significantly different from NT group at $P < 0.05$, *Tg+/Con group significantly different from NT group at $P < 0.05$. All data are means (\pm S.E.M.).

A β Histopathology

At around 15 months into A β immunotherapy (and at 17 months of age), A β immunostaining and Congo Red staining were performed on brain sections from the frontal cortex, dentate gyrus and both CA1 and CA3 regions of the hippocampus in Tg+ mice. The results shown in Table 5 illustrate that long-term A β immunotherapy did not alter total or compact A β deposition in any of the brain regions analyzed. However, one of the four Tg+/A β mice did have appreciable reductions in mean A β levels ranging from 76 to 86% compared to Tg+/Con mice.

Table 5. Total A β (diffuse and compact) and compact A β (Congo Red) for Tg+/Con (n=4) and Tg+/A β (n=4) at 17 months of age within frontal cortex and hippocampus

| Brain Area | Tg+/Con | Tg+/Aβ |
|------------------------------------|------------------|--------------------------------|
| Total Aβ | | |
| Frontal Cortex | 41.66 \pm 3.96 | 34.07 \pm 10.38 |
| Hippocampal CA1 | 52.09 \pm 2.27 | 41.92 \pm 12.16 |
| Hippocampal CA3 | 42.25 \pm 0.89 | 34.00 \pm 9.49 |
| Dentate Gyrus | 44.88 \pm 1.34 | 39.23 \pm 11.95 |
| Compact Aβ | | |
| Frontal Cortex | 2.02 \pm 0.238 | 1.91 \pm 0.28 |
| Hippocampal CA1 | 2.56 \pm 0.29 | 2.11 \pm 0.34 |
| Hippocampal CA3 | 1.47 \pm 0.10 | 1.79 \pm 0.11 |
| Dentate Gyrus | 1.59 \pm 0.52 | 1.21 \pm 0.10 |

FA and Correlation Analyses

Table 6 shows the factor analysis of behavioral measures with and without A β histopathologic measures in order to determine the underlying relationships between such behavioral measures and A β deposition. At the adult (4½-6 months) test point, of the 19 behavioral measures analyzed, 15 measures loaded on three principle factors which together account for more than 70% of the total variance. All measures of RAWM and Morris Water maze loaded heavily under factor 1, which was clearly cognitively-based and accounted for more variance (32.5%) than either of the other two factors. In contrast, factor 2 was strongly sensorimotor/anxiety based, including activity/exploratory, elevated plus maze and string agility measures. At the aged (15-16½ month) test point, many of the above 19 behavioral measures loaded into five principle factors (Table 6). Similar to the adult test point, factor one was largely cognitively based and factor 2 was primarily sensorimotor/anxiety based. Factor 1 again included all measures of RAWM and Morris water maze in addition to both measures of platform recognition; this factor, therefore, encompassed working memory, reference learning/memory and recognition/identification. Also, both measures of platform recognition loaded separately into factor 3. Sensorimotor/ anxiety-based factor 2 retained three measures present at the earlier time point and gained an additional elevated plus-maze measure (number of closed arm entries). Two other measures (time in plus-maze open arms and string agility) which previously loaded together in factor 2 at the adult test point, loaded separately into factors 4 and 5, respectively. Inclusion of the eight histopathologic measures at the aged test point resulted in five of these measures loading into factor 1 with essentially all of the RAWM and Morris water maze measures. The five histopathologic measures in factor 1

included all four brain areas stained for “total” A β and one area that was stained for compact A β deposition. Compact A β deposition in region CA3 of the hippocampus loaded with various sensorimotor and cognitive-based measures in factor 2. Factor 3 was largely activity/exploratory-based, while factor 4 included only Congo Red staining in the cortex and dentate gyrus. Lastly, factor 5 solely contained the time in plus-maze open arms. Therefore, similar factor loadings were seen with and without A β histopathology, although the pathologic measures did modify the behavioral loadings somewhat.

At the aged test point, correlation analyses were performed between the nine cognitive-based measures from factor 1 and all eight of the A β histopathologic measures. This analysis involved all Tg⁺ mice (4 Tg⁺/A β mice and 4 Tg⁺/Con mice). Of a total of 72 total correlations that were done, there were no significant correlations found.

Table 6. Factor Loadings of behavioral measures, with and without pathologic measures.

| Factor | 4½-6 Months | 15-16½ Months without Aβ measures | 15-16½ Months with Aβ measures |
|--------|--|--|--|
| 1 | (32.46) RM-T4-Fin RM-T5-Fin RM-T4 RM-T5 WM-Avg WM-Fin WM-Ret | (36.35) RM-T4-Fin RM-T5-Fin RM-T4 RM-T5 WM-Avg WM-Fin WM-Ret PR-Avg PR-Fin | (32.53) RM-T4-Fin RM-T5-Fin RM-T4 RM-T5 WM-Avg WM-Ret Aβ-CX Aβ-CA1 Aβ-CA3 Aβ-DG CR-CA1 |
| 2 | (27.80) OF YM-Ent EP-#O EP-TO STR PR-Avg | (19.50) OF YM-Ent EP-#O EP-#C YM-Alt | (18.80) PR-Avg PR-Fin YM-Ent STR CPE-Avg CR-CA3 |
| 3 | (10.41) CPL-Avg EP-#C | (14.06) PR-Avg PR-Fin | (18.24) OF EP-#O EP-#C WM-Fin |
| 4 | | (7.87) EP-TO | (10.94) CR-CX CR-DG |
| 5 | | (6.07) STR | (8.96) EP-TO |

Note: Percent of total variance explained by a given factor is indicated in bold type within parentheses. Measures from the same task and pathologic markers are color-coded to facilitate identification.

Aβ-CA1 = total Aβ within region CA1; Aβ-CA3 = total Aβ within region CA3; Aβ-DG = total Aβ within dentate gyrus; CR-CA1 = Congo Red staining region CA1; CR-CA3 = Congo red staining region CA3; CR-DG = Congo Red staining dentate gyrus; EP-#C = # closed arm entries in elevated plus maze; EP-#O = # open arm entries in elevated plus maze; EP-TO = % time in open arm in elevated plus maze; PR-Avg = platform recognition overall average; PR-Fin = platform recognition final day average; RM-T4 = radial arm water maze errors overall trial 4; RM-T5 = radial arm water maze errors overall trial 5; RM-T4-Fin = radial arm water maze errors last block trial 4; RM-T5-Fin = radial arm water maze errors last block trial 5; WM-Avg = water maze latency overall average; WM-Fin = water maze latency last day; WM-Ret = water maze % time spent in Q2 during probe trial

DFA

Discriminant function analysis (DFA) was used in order to determine if the behavioral performance of each of the three groups (NT, Tg+/Con and Tg+/A β) could be distinguished from each other at both 4½-6 month and 15-16½ month test points. Table 1.4 shows a summary of two DFA methods including the “direct entry” method (including all behavioral measures) and the “step-wise forward” method (selects behavioral measures from all the measures based on their contribution to the variance) at both test points. The direct entry DFA could not distinguish the three groups at either test point based on their behavioral performance. However, at the 4½-6 month test point, the step-wise forward DFA could completely distinguish between all three groups such that their rank order of performance was as follows: NT>Tg+/A β >Tg+/Con. Additionally, at the 15-16½ test point, the step-wise forward DFA successfully distinguished between the two Tg+ groups, even with the lesser number of animals. At both test points a sensorimotor measure (Y-maze entries) and a cognitive-based measure (either RAWM trial 5 on last block or platform recognition averaged latency) were retained as providing the maximal discriminability.

Additional DFAs were performed utilizing only the cognitive-based measures that loaded on factor 1 in FA (see Table 1.3). The direct entry DFA at the adult test point resulted in significant discrimination between the NT group and both Tg+ groups using all seven cognitive measures in factor 1. In contrast, direct entry DFA at the aged test point was unable to successfully distinguish between any of the three groups using the nine behavioral measures from the factor 1. However, all three groups were completely

distinguished from each other at the adult test point by step-wise forward DFA (rank order by performance was NT>Tg+/A β >Tg+/Con). The measures retained as providing maximal discrimination were cognitive-based, including measures from RAWM and Morris water maze. Similar to the adult test point, step-wise forward DFA at the aged test point could successfully discriminate the NT group from the Tg+/Con group. More importantly, step-wise forward DFA could not distinguish the NT group from the Tg+/A β group based on their behavioral performance. Three measures, including two from RAWM and one from platform recognition, were retained as providing maximum discrimination between the NT group and the Tg+/Con group.

Fig. 18 shows canonical scores plot of both the seven- and nine-measure step-wise forward DFAs at 4½-6 month and 15-16½ test points, respectively. The measures that provided the maximal discrimination between groups were RAWM trial 5 performance overall and RAWM trial 5 performance on the last block of testing (Table 7). Between 85-88% of the mice at the adult test point and 69-75% of the mice at the aged test point were correctly classified by the step-wise forward DFA.

Table 7. Summary of discriminant function analyses.

| Measures | Time Point | Direct Entry Method (All Measures Used) | Stepwise-Forward Method | |
|------------------------------------|------------|--|------------------------------|------------------------------------|
| | | | Significance | Measures Retained |
| All 19 | 4½-6M | N.S. | p < 0.0005 (85%) | RM-T5 Fin YM-Ent |
| All 19 | 15-16½M | N.S. | p < 0.02 *B vs C (75%) | PR-Avg YM-Ent |
| Factor 1 (7 cognitive measures) | 4½-6M | p < 0.0025 *A vs B *A vs C (53%) | p < 0.0005 (88%) | RM-T4 RM-T5 WM-Avg WM-Ret |
| Factor 1 (9 cognitive measures) | 15-16½M | N.S. | p < 0.02 *A vs B (69%) | RM-T5 RM-T5 Fin PR-Avg |

Notes:

(1) p-values are from Wilks's lambda.

(2) Post-hoc analysis revealed complete discrimination between groups, except where otherwise indicated.

* Group codings: A = non-transgenic, B = Tg+/Con, C = Tg+/Aβ

(3) Percent correct classification (from Jackknifed matrix) indicated in parenthesis.

Abbreviation: N.S. = not significant; all other abbreviations defined in Table 1.3.

Canonical Score Plots

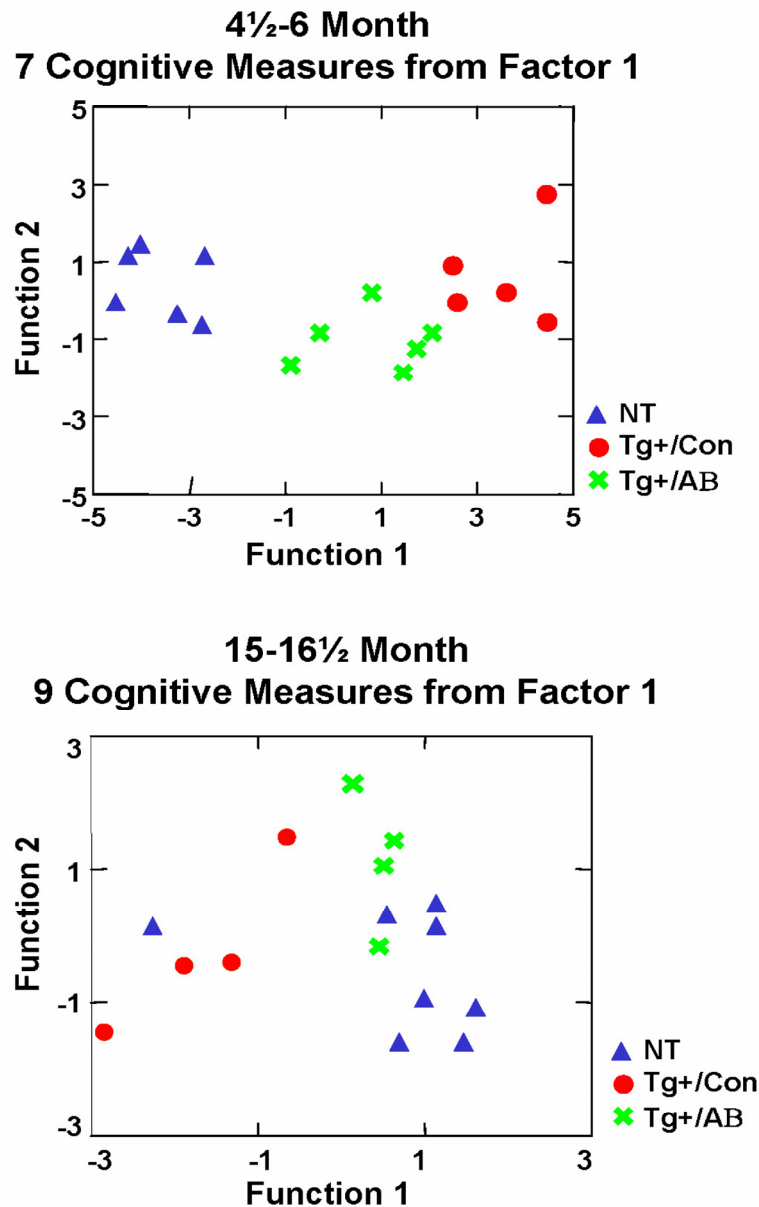


Fig. 18. Canonical score plots of step-wise forward discriminant function analyses used to compare the “overall” cognitive performance of NT, Tg+/Con, and Tg+/A β groups. Each symbol represents the cognitive performance of one animal graphed from the two linear functions derived in the DFA. At the 4½-6 month test point involving all seven cognitive measures from factor 1 (upper), all groups could be distinguished from one another in cognitive performance (rank order: NT>Tg+/A β >Tg+/Con). At the 15-16½ month test point involving all nine cognitive measures from factor 1 (lower), the performance of the NT and Tg+/A β mice could not be distinguished from one another, while NT mice were clearly distinguishable (better than) Tg+/Con mice.

II. Omega-3 Fatty Acid Study- Survival Analyses

Fig. 19 shows profound differences in the survivability of F1 generation APP/PS1 (n=13) and PS1 (n=7) mice that were fed the omega-3-deficient (omega-6 only) diet beginning at 6 weeks of age. All of the PS1 mice survived on this diet for 120 days, at which time the mice were euthanitized. In sharp contrast, the APP/PS1 mice experienced 100% mortality, mostly within 20 days of beginning the diet. This resulted in a highly significant difference in the cumulative proportion surviving, as determined by the Cox-Mantel test ($P < 0.0001$).

In addition to the survival analysis within the F1 generation, F2 generation APP (n=7), PS1 (n=9) and NT (n=9) were started on the omega-3-deficient diet at an average of 6 weeks of age. As shown in Fig. 20, all genotypes showed similar survival on the experimental diet ($P > 0.05$), with the majority of the mortality occurring between 5 and 10 days after beginning the diet.

By comparing survivability within the PS1 transgenic groups for F1 versus F2, it is evident that it is a combination of the interaction between the PS1 transgene and the background strain that determines survival on the omega-3-deficient diet (Fig. 21). There is a significant difference in the cumulative proportion surviving ($P < 0.0001$), with the F1 PS1 mice having complete survival in contrast to the 100% mortality seen in the F2 generation of PS1 mice. In reference to the difference in the background between generations, the F1 generation was composed of a higher amount of B6 with a lesser amount of C57 present as compared to the F2 generation mice. This suggests that the interaction between the B6 background and the PS1 transgene provided limited

protection against a diet that completely lacks omega-3 fatty acids.

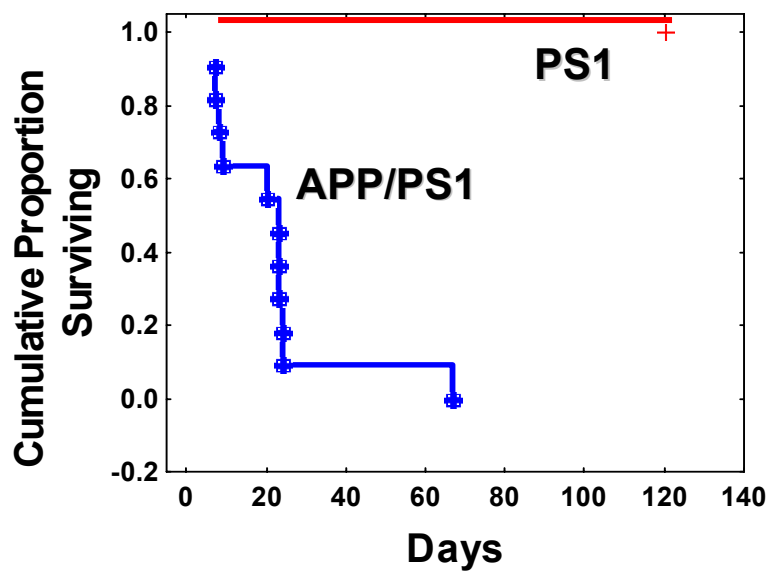


Fig. 19. Survivability analysis showing days on the omega-3-deficient diet wherein the F1 PS1 mice had no mortality through 3 months after starting on the experimental diet. However, the F1 APP/PS1 mice had no survival on the omega-3-deficient diet.

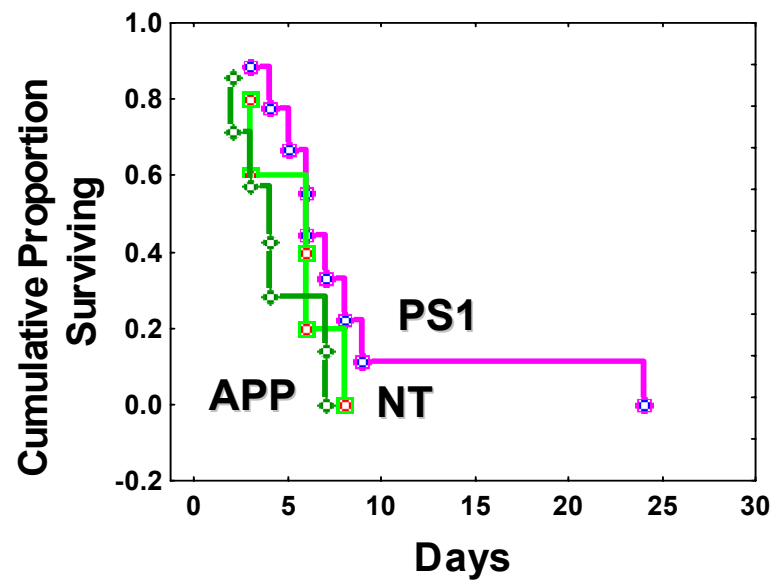


Fig. 20. Survivability analysis showing days on the omega-3-deficient diet. All 3 groups of the F2 mice had similar mortality after starting on the experimental diet.

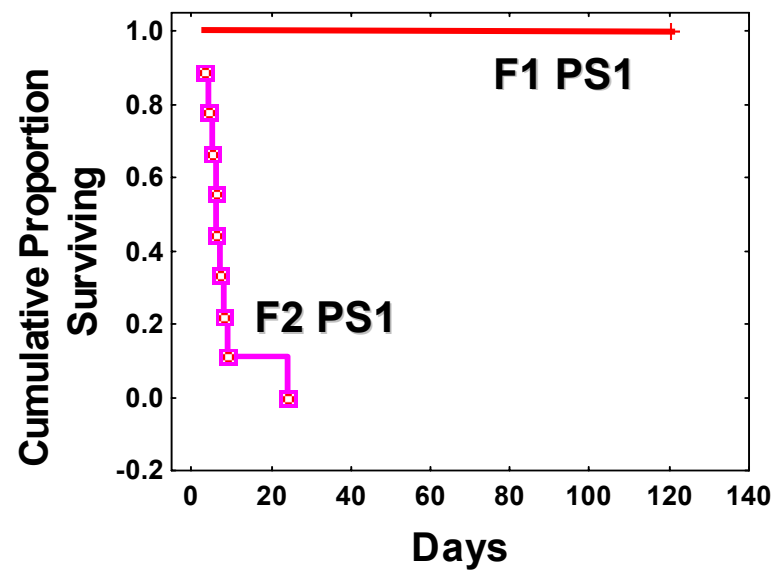


Fig. 21. Survivability analysis comparing F1 PS1 mice to F2 PS1 mice, showing a significant difference in survival between the two generation within the same genotype. As shown, the PS1 transgene is not completely protective against mortality on a diet that is devoid of omega-3 fatty acids.

III. Omega-3 Fatty Acid Study: Behavioral, Pathologic, and Neurochemical Assessments

Following a pre-diet baseline determination of body weight, all mice were weighed weekly throughout the 5½-7 month duration of the study. Averaging all weekly weight determinations, no overall differences were found between any of the six groups of mice, nor were there any differences in final weights at the end of the study (data not shown).

Significant differences in behavioral performance due to transgenicity (standard diet NT vs. standard diet Tg+) or diet are summarized in Table 8. Table 9 presents differences in behavioral performance due to transgenicity or diet (standard vs. high n-3) for only APP/PS1 and NT mice; all APP mice and also all high n-6 diet groups were excluded.

Table 8. A summary of transgenic and dietary behavioral effects (high n-3 and high n-6) in NT and Tg+ mice.

| Task | Transgenic Effect | Diet Effect in Tg+ mice (vs. Standard NT) | Diet Effect in Tg+ mice (vs. Standard Tg+) |
|--------------------------------------|-------------------|--|---|
| Open Field Activity | | ↑ Tg+n-3 | ↑ Tg+n-3 |
| Balance Beam | Tg+S Impaired | Tg+n-3 & Tg+n-6 impaired | |
| String Agility | Tg+S Impaired | Tg+n-3 & Tg+n-6 not impaired | |
| Elevated Plus Maze | | | |
| % open arms | | | |
| Open arm entries | | | |
| Closed arm entries | | | ↑ Tg+n-3 & Tg+n-6 |
| Y-maze | | | |
| Arm entries | | ↑ Tg+n-3 | ↑ Tg+n-3 |
| % Alternation | | | |
| Morris Maze Acquisition | | | |
| Morris Maze Retention | Tg+S Impaired | Tg+n-6 not impaired | |
| Circular Platform Acquisition | | Tg+ n-6 impaired | Tg+n-6 impaired |
| Platform Recognition | Tg+S Impaired | Tg+n-6 impaired; Tg+n-3 not impaired | Tg+n-6 impaired |
| Radial Arm Water Maze | | | |
| Overall | Tg+S Impaired | Tg+n-3 & Tg+n-6 impaired | |
| Last Block | Tg+S Impaired | Tg+n-3 & Tg+n-6 not impaired | |

Table 9. A summary of transgenic and dietary behavioral effects (high n-3 vs. standard diet) in NT and APP/PS1 mice.

| Task | Transgenic Effect | Diet Effect (vs. Standard NT) | Diet Effect (vs. Standard Tg+) |
|--------------------------------------|-------------------|-------------------------------|--------------------------------|
| Open Field Activity | | ↑ Tg+n-3 | ↑ Tg+n-3 |
| Balance Beam | Tg+S Impaired | | |
| String Agility | Tg+S Impaired | Tg+n-3 not impaired | |
| Elevated Plus Maze | | | |
| % open arms | | | |
| Open arm entries | | | |
| Closed arm entries | | | ↑ Tg+n-3 |
| Y-maze | | | |
| Arm entries | | ↑ Tg+n-3 | ↑ Tg+n-3 |
| % Alternation | | | |
| Morris Maze Acquisition | | | |
| Morris Maze Retention | Tg+S Impaired | Tg+n-3 not impaired | |
| Circular Platform Acquisition | | | |
| Platform Recognition | Tg+S Impaired | | Tg+n-3 Improved |
| Radial Arm Water Maze | | | |
| Overall | Tg+S Impaired | Tg+n-3 not impaired in errors | |
| Last Block | Tg+S Impaired | Tg+n-3 not impaired | |

Sensorimotor- and Anxiety-Based Tasks

For all sensorimotor- and anxiety-based tasks, there were no effect of high n-3 or high n-6 diets on performance of NT mice (Figs. 22-24). Thus, neither diet affected sensorimotor abilities or level of anxiety in NT mice. Some effects of transgenicity and diet were, however, evident for Tg+ mice, as indicated below.

Open Field and Y-maze Entries. The Tg+ high n-3 mice had increased open field activity (Fig. 22a) compared to all NT groups ($P<0.01$) and Tg+ standard mice ($P<0.05$). Similarly, within the Y-maze task for activity/exploration, arm entries of the Tg+ high n-3 group were elevated compared to all NT groups ($P<0.01$) and Tg+ standard mice ($P<0.02$) (Fig. 24a). Thus, a high n-3 diet increased activity/exploration in Tg+ mice.

Balance Beam. In the balance beam task (Fig. 22b), all three groups of Tg+ mice exhibited equally poor balance ability compared to NT standard-fed mice, as indicated by short trial times prior to falling from the beam. A strong overall effect of transgenicity was present [$F(1,47)=22.47$; $p<0.0001$]. These results indicate there was no beneficial effect on balance for either high n-3 or n-6 diets in Tg+ mice.

String Agility. Although a significant overall effect of transgenicity was evident ($p<0.002$, Mann-Whitney U test), neither of the Tg+ groups fed the high n-3 or high n-6 diets exhibited deficits in string agility compared to NT standard-fed mice (Fig. 22c). In contrast, the standard Tg+ mice did show a deficit in string agility compared to standard NT mice ($P<0.05$). Nonetheless, all 3 Tg+ groups showed the same level of agility.

Elevated Plus-Maze. In the elevated plus-maze task, there were no significant differences in either the percent of time spent in the open arms or the number of open arm

entries between any of the Tg⁺ and NT groups (Fig. 23a and b). As well, there were no overall effects of transgenicity for either of these measures. Despite the lack of difference in open arm entries, both the high n-3 and high n-6 had increased closed arm entries compared to the standard Tg⁺ mice, but not vs. NT standard-fed mice (Fig. 23c). Within the NT mice, there was no difference in closed arm entries between any of the diet groups.

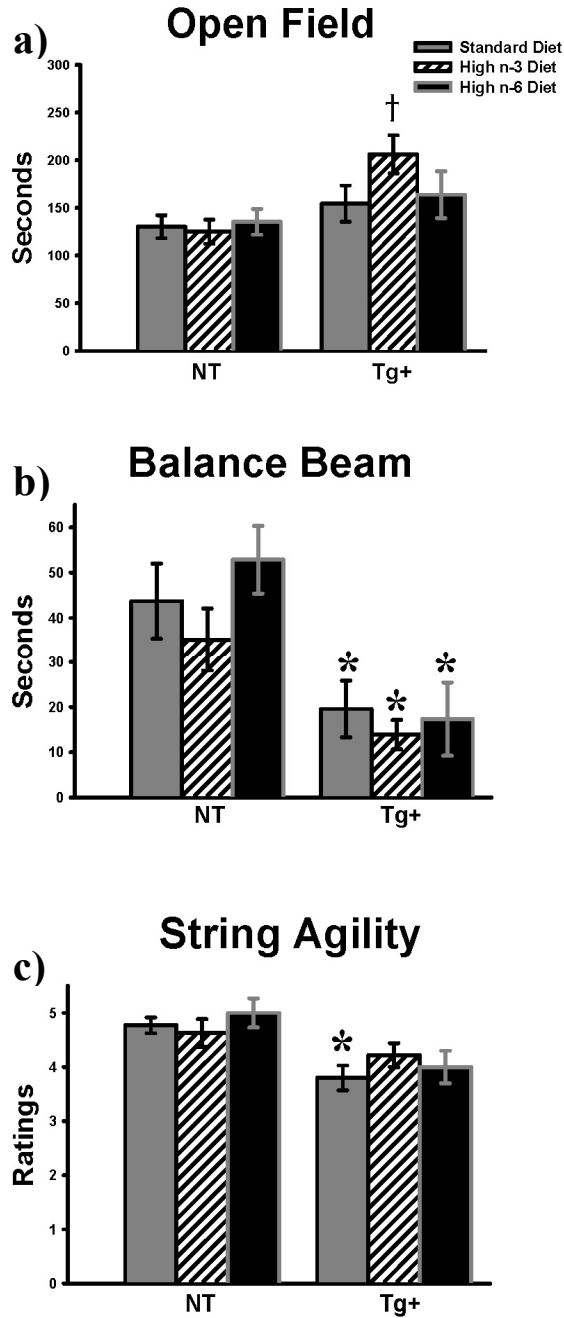


Fig. 22. Comparison of sensorimotor function in NT and Tg+ mice fed either standard, high n-3 or high n-6 diets. Data represent the mean \pm S.E.M. (a) Open field activity, determined by open field line crossings, was increased in high n-3 Tg+ mice. (b) Equilibrium/agility, as measured by time on a balance beam, was impaired in all three Tg+ diet groups. (c) String agility, as measured by forepaw grip suspension was impaired only in Tg+ standard mice, although there was no difference in agility between Tg+ groups. *Significantly different from NT standard group, with $P < 0.05$ or higher level of significance. †Significantly different from all NT mice and standard Tg+ mice.

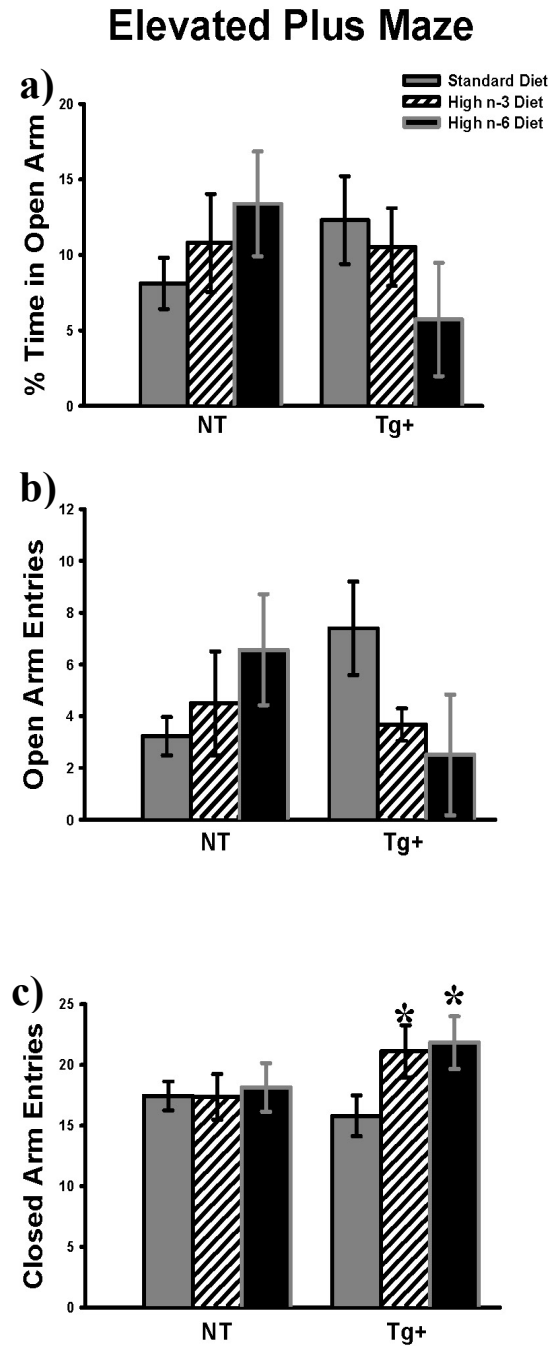


Fig. 23. Anxiety/emotionality, as determined in the Elevated Plus-maze by percent time in open arms and arm entries. No differences were evident in either percent time in open arms (a) or number of open arm entries (b) within any of the NT and Tg+ groups. However, both high n-3 and high n-6 Tg+ mice had an increased number of closed arm entries vs. standard Tg+ mice. Data represents the mean \pm S.E.M. * Significantly different from standard Tg+ mice at $P < 0.05$.

Cognitive-based Tasks

As shown in Figures 24 – 37, there was no effect of a high n-3 diet on performance of NT mice in any of the cognitive-based tasks. This was also the case for NT mice on a high n-6 diet, with the exception of n-6 induced deleterious effects on Morris maze retention and circular platform errors. Thus, these diets had minimal impact on cognitive performance in normal mice. For Tg+ mice, however, there were some effects of diet on cognitive performance. These effects were largely only seen in comparison to standard NT mice, but not standard Tg+ mice.

Y-Maze Alternation. In Y-maze testing for spontaneous alternation, there was no overall effect of transgenicity, nor were there any differences among the NT and Tg+ groups (Fig. 24b).

Morris Water Maze Acquisition. In addition to the lack of impairment of Tg+ mice in Y-maze spontaneous alternation, there also was no transgenic impairment in Morris water maze acquisition over 10 days of testing. For both 5-day blocks, all NT groups and all Tg+ groups performed similarly to each other (Fig. 25). Even on the final day of testing, there were no group differences in acquisition (data not shown).

Morris Water Maze Retention. Within the NT group, the standard and high n-3 diet mice, but not the high n-6 mice, showed an exclusive quadrant preference; nonetheless, all three NT groups had a similar number of annulus crossings. Despite no impairment in Morris maze acquisition, standard Tg+ mice showed reference memory impairment compared to standard NT mice in both time spent in the former platform-containing quadrant and annulus crossings (Fig. 26). Surprisingly within the Tg+ groups, only the high n-6 Tg+ mice had an exclusive quadrant preference, whereas the high n-3

Tg⁺ mice only showed a partial quadrant preference for Q2 versus Q1. Similarly, both the standard and high n-3 Tg⁺ mice exhibited a lower number of annulus crossings during the memory retention trial compared to the standard NT mice. However, there were no differences in annulus crossing when the 3 Tg⁺ groups were compared directly.

With inclusion of only APP/PS1 mice in both standard and high n-3 groups (Fig. 27), only the standard Tg⁺ group remained completely impaired in memory retention versus standard NT mice – this, for both quadrant preference and annulus crossings. In contrast, the high n-3 APP/PS1 mice showed a partial quadrant preference for Q2 compared to Q1 and Q4. Consistent with this modestly improved retention of n-3 APP/PS1 mice, they also exhibited a similar number of annulus crossings compared to standard NT mice (Fig. 27). By contrast, standard APP+PS1 mice exhibited a lower number of annulus crossings vs. standard NT mice. The only modest benefit of a high n-3 diet in APP+PS1 mice is underscored by the fact that there was no difference in annulus crossings between APP+PS1 mice on the high n-3 diet vs. those on a standard diet. Consistent with their lack of quadrant preference, APP/PS1 standard mice exhibited a lower number of annulus crossings vs. standard NT mice.

The Morris maze retention data suggest that both n-3 and n-6 fatty acid supplementation can partially correct memory retention deficits that are otherwise present in standard-fed Tg⁺ mice, although the high n-6 diet was actually deleterious in NT mice. During the probe trial, there were no differences in swim speed among the Tg⁻ and Tg⁺ groups.

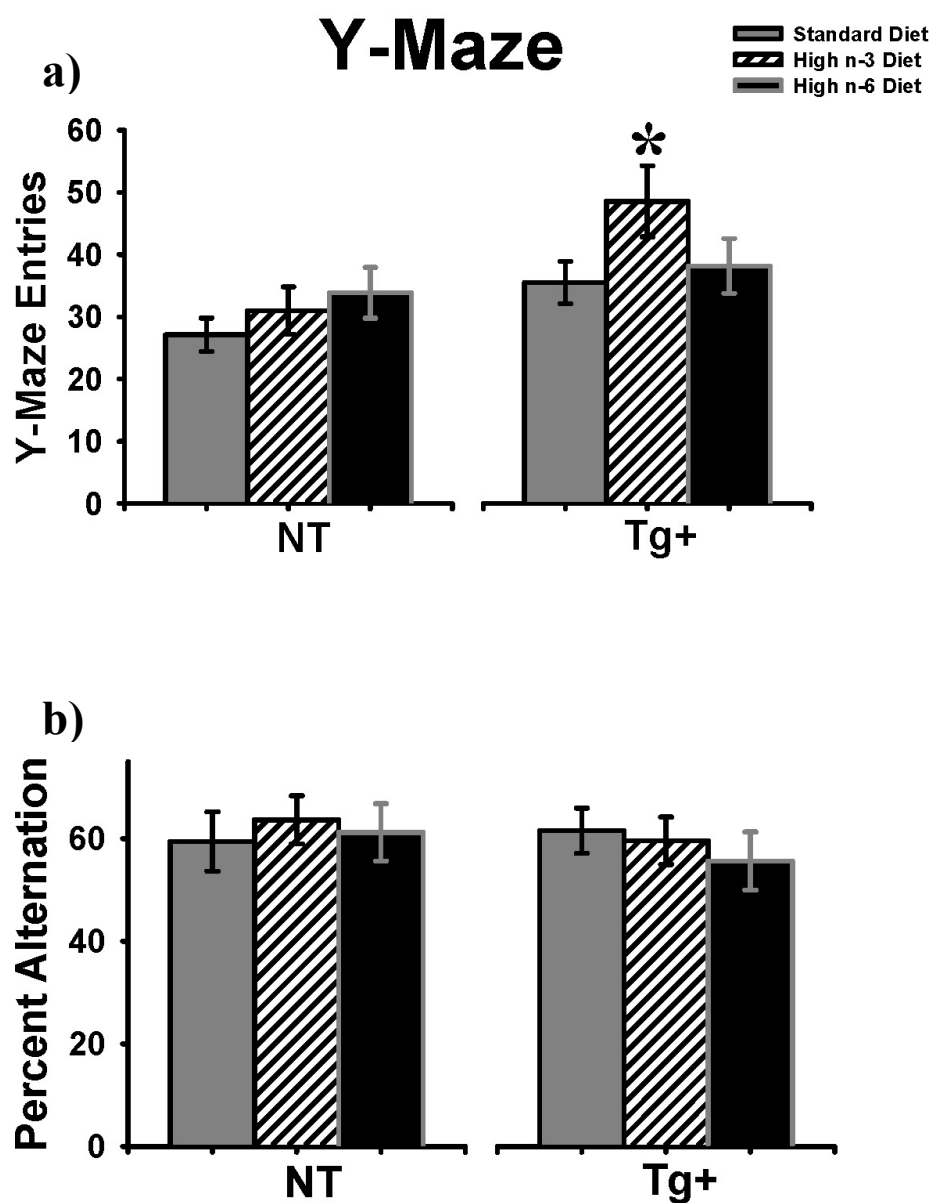


Fig. 24. Y-maze arm entries (a) and percent spontaneous alternation (b) for standard, high n-3 and high n-6 diet groups within NT and Tg+ mice. Data represents the mean \pm S.E.M. (a) High n3 Tg+ mice had significantly more arm entries (e.g., increased activity) compared to all three NT groups and standard Tg+ mice. (b) There were no group differences for either NT or Tg+ groups in percent spontaneous alternation. *Significantly different from standard-fed Tg+ mice at $P < 0.02$.

Morris Maze Acquisition

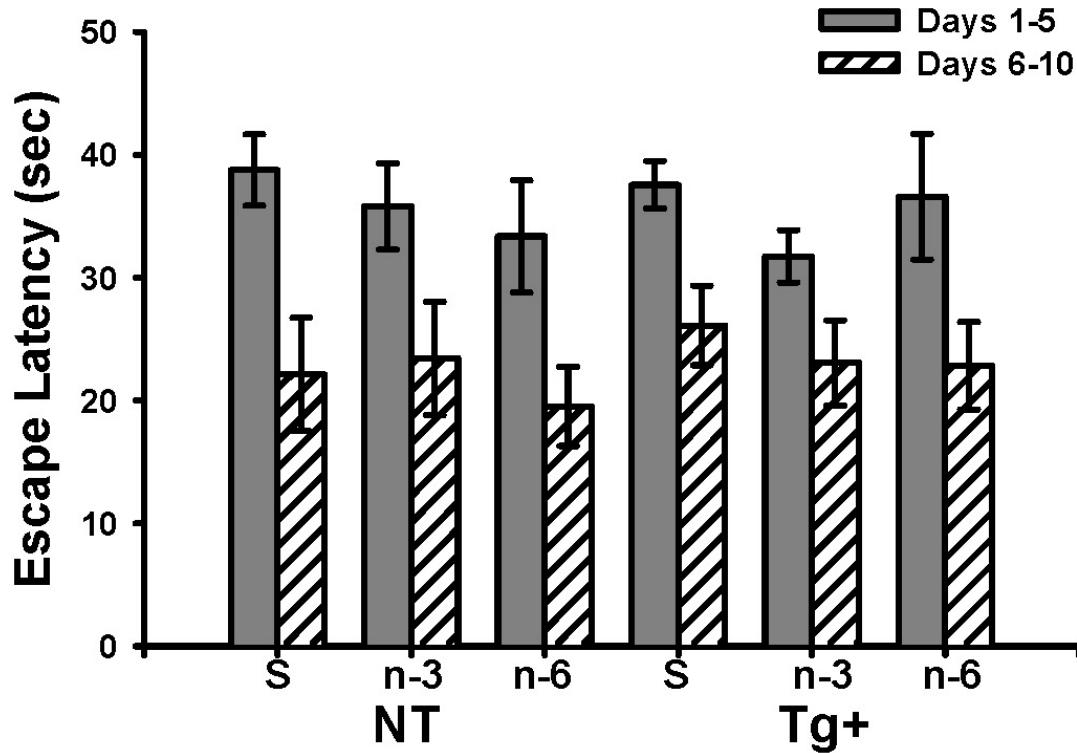


Fig. 25. Morris water maze acquisition for standard, high n-3 and high n-6 diet groups of NT and Tg+ mice. The 10 days of acquisition, as measured by latency to find a submerged stationary platform, are presented in two 5-day blocks. For both blocks and for all three NT and three Tg+ groups, there were no differences in acquisition.

Morris Maze Retention

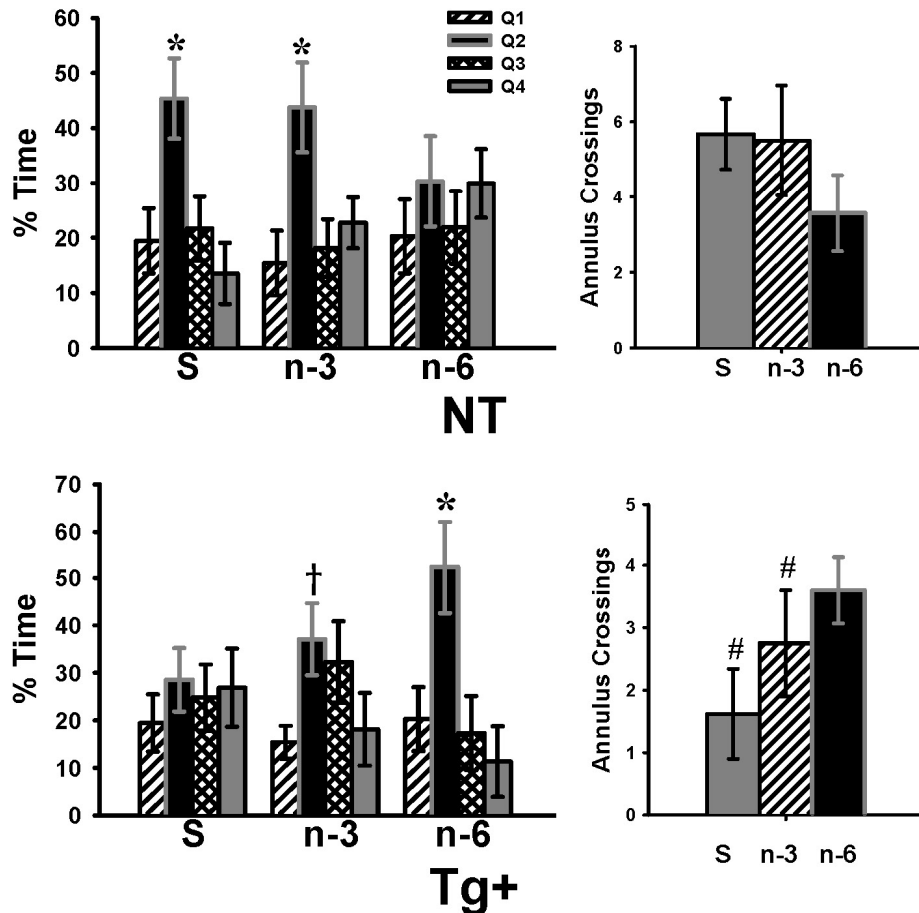


Fig. 26. Probe trial testing for reference memory retention, assessed on the day following the completion of water maze acquisition. A single 1-min trial was done, with the percent of time spent in the quadrant formerly containing the submerged platform (Q2) and the number of annulus crossings determined. Within the NT groups, both the standard-fed and high n-3 mice showed an exclusive quadrant preference for Q2 compared to all other quadrants. In sharp contrast, the high n-6 NT mice showed no quadrant preference. Within the Tg+ mice, the standard-fed mice showed no quadrant preference, while the high n-3 mice showed a partial quadrant preference for Q2 compared to Q1 († = significantly higher than Q1 at $P < 0.05$). Also, the high n-6 Tg+ mice showed a significant exclusive quadrant preference for Q2 versus all other quadrants. (* = significantly higher than all other quadrants at $P < 0.05$ or higher level of significance). No differences were seen in number of annulus crossings within the NT mice, however the standard and high n-3 Tg+ had significantly fewer annulus crossing compared to standard NT mice (# significantly different from standard NT at $P < 0.05$), although there were no differences in annulus crossings among the 3 Tg+ groups.

Morris Maze Retention

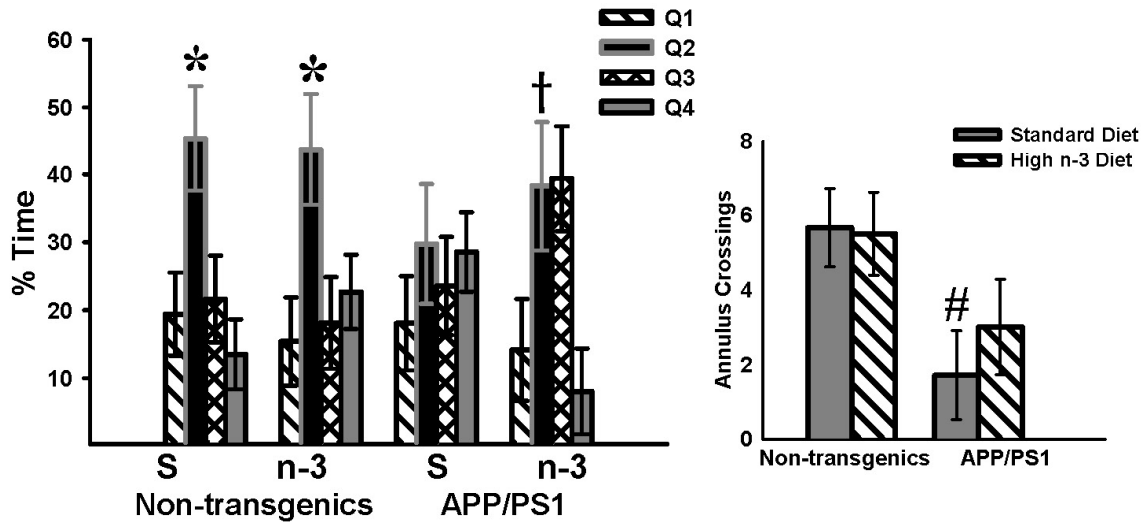


Fig. 27. Probe trial testing for reference memory retention, assessed on the day following the completion of water maze acquisition, including only NT and APP/PS1 groups. Within the NT groups, both the standard-fed and high n-3 mice showed an exclusive quadrant preference for Q2 compared to all other quadrants (* significantly higher than all other quadrants at $p < 0.05$ or higher level of significance). Within the APP/PS1 mice, the standard-fed mice showed no quadrant preference, while the high n-3 mice showed a partial quadrant preference for Q2 compared to Q1 and Q4 († = significantly higher than Q1 and Q4 at $P < 0.05$ and $P < 0.02$, respectively). No differences were seen in number of annulus crossings within the NT mice, however the standard APP/PS1 mice had significantly fewer annulus crossing compared to standard NT mice (# significantly different from standard NT at $P < 0.02$), while there was no difference between the high n-3 APP/PS1 mice and standard NT mice.

Circular Platform. Assessment of spatial learning/memory in the circular platform task showed marked differences between the diet groups on the last day of this 8-day task. Although there was no transgenic effect in either number of head pokes/errors into non-escape holes or latency to find the escape hole, there were dietary differences within the NT and Tg+ groups. Fig 28 shows that the high n-6 NT mice had impaired spatial learning/memory, as evidenced by increased errors compared to both standard NT mice and high n-3 NT mice. However, there were no differences within the NT groups in latency. Within the Tg+ groups, the high n-6 mice were also impaired in errors (vs. standard Tg+ mice) and latency (vs. standard NT mice) to find the escape hole. For both NT and Tg+ genotypes, high n-3 mice performed similar to standard fed mice. Since there were no differences in performance between genotypic groups for any diet, the two genotypes were combined. Fig 29 shows the resultant strong effect of diet in the circular platform task. Together, NT and Tg+ mice fed the high n-6 diet had increased errors compared to both standard and high n-3 diet groups. Similarly, the combined high n-6 diet group had increased latency compared to the combined standard diet group. These data indicate that a diet high in n-6 fatty acids induces impairment in spatial reference learning/memory, while a diet high in n-3 fatty acids does not induce such an impairment.

Circular Platform Last Day

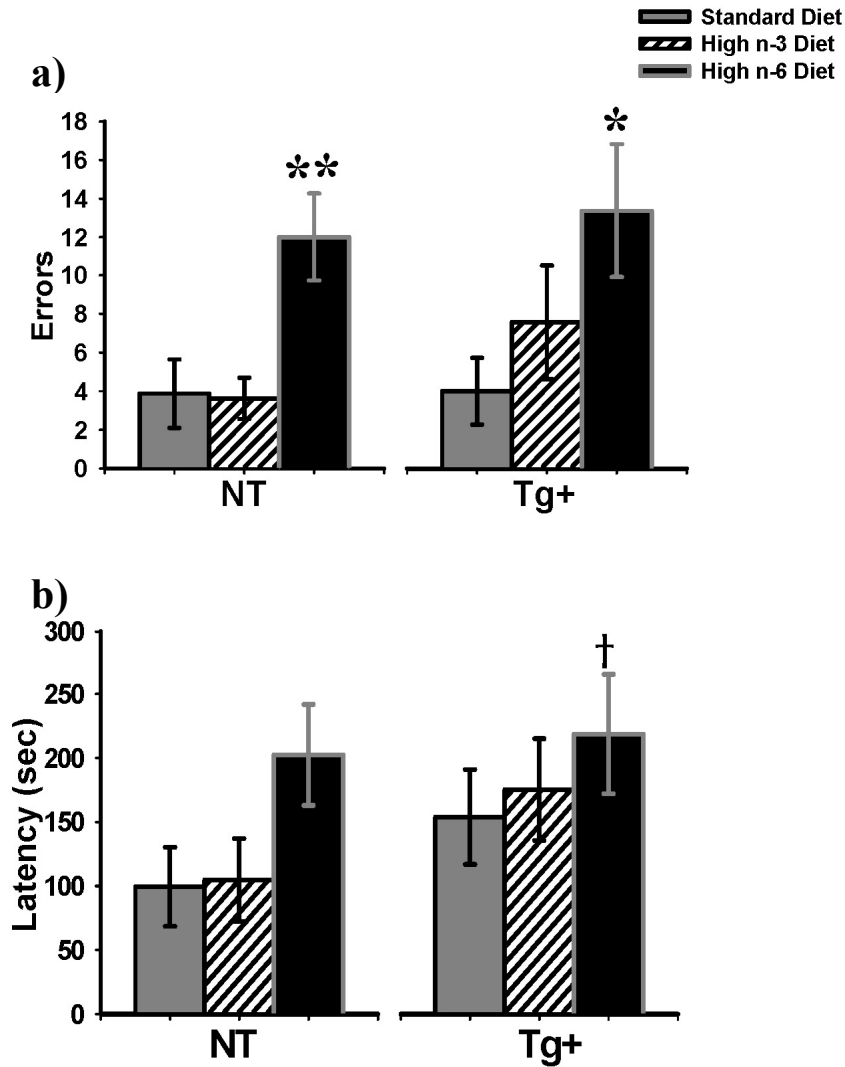


Fig. 28. Spatial learning/memory, as determined by the circular platform task by number of head pokes/ errors (a) into non-escape holes and latency (b) to find the escape hole across 8 days of testing, with the last day of testing shown above. (a) High n-6 NT mice showed impairment by an increase of errors compared to both standard and high n-3 mice (** significantly different from standard and high n-3 NT mice at $P < 0.02$). Similarly, high n-6 Tg+ mice had increased errors compared to standard Tg+ (* $p < 0.05$), with the high n-3 Tg+ mice having similar performance to the standard Tg+ group. (b) Despite a difference in errors, there were no differences between the NT mice with respect to latency to find the escape hole. However, the high n-6 Tg+ were impaired in latency compared to the standard NT mice († significantly different from standard NT mice at $P \leq 0.05$).

Circular Platform

Combined Genotypes

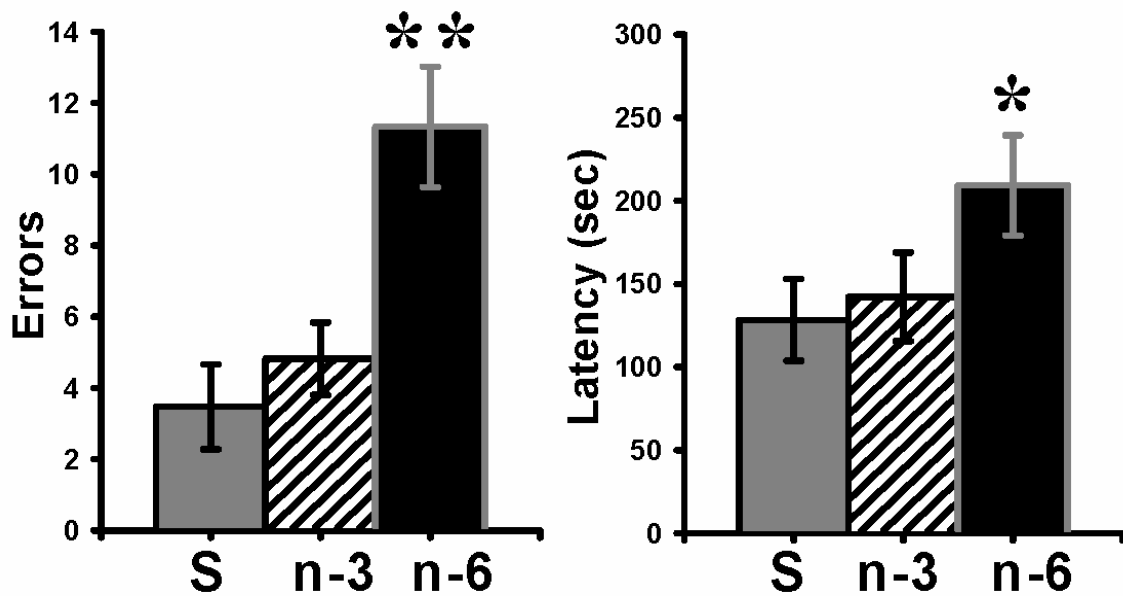


Fig 29. The last day of circular platform shows a strong effect of diet by combining both genotypes. Together, NT and Tg+ mice on the high n-6 diet were impaired, as shown by a high number of errors into non-escape holes versus both standard and high n-3 groups (** = $P < 0.01$ or higher level of significance). Also, the high n-6 mice had increased latency to find the escape hole compared to the standard diet group (* = $P < 0.05$).

Platform Recognition. Within the NT groups, all mice collectively showed a strong learning effect across the four days of testing, with no differences between the diet groups overall or at individual days (Fig. 30a). In contrast, the standard Tg⁺ mice were impaired overall versus the standard NT mice (Fig. 30b; $p < 0.05$). Even in comparison to the standard Tg⁺ group's poor performance, high n-6 Tg⁺ were significantly worse than all five other groups, including both standard and high n-3 Tg⁺ mice. In contrast to the impaired high n-6 Tg⁺ performance, the high n-3 Tg⁺ group performed significantly better overall ($p < 0.005$). Although performance of high n-3 Tg⁺ mice was no different from standard NT mice overall, their performance still was not improved enough to be significantly better than standard Tg⁺ mice. High n-3 Tg⁺ mice did have significantly lower latencies on days 2, 3, and 4 versus high n-6 Tg⁺ mice. The platform recognition data indicate that a diet high in n-3 fatty acids protects AD transgenic mice from impairment in recognition/identification. In addition, a diet with a high amount of n-6 fatty acids induces an impairment in recognition/identification that is even greater than that normally present in Tg⁺ on a standard diet.

Inclusion of only APP/PS1 mice for both standard and high n-3 Tg⁺ groups revealed marked differences in recognition/identification within this task. Standard APP/PS1 mice were impaired overall ($p < 0.02$) and specifically on days 3 and 4 versus the standard NT mice (Fig. 31b; $p < 0.02$). In sharp contrast to the impaired standard APP/PS1 performance, the high n-3 APP/PS1 group performance was no different from the standard NT mice on all days and over all 4 days of testing. Moreover, by day 4 of the task, the high n-3 APP/PS1 mice performed significantly better (had lower latency) than the standard APP/PS1 and were no different from standard NT mice. These data

indicate that a diet high in n-3 fatty acids protects AD transgenic mice from impairment in recognition/identification and by the end of the task fully reversed the transgenic impairment present in standard-fed mice.

Platform Recognition

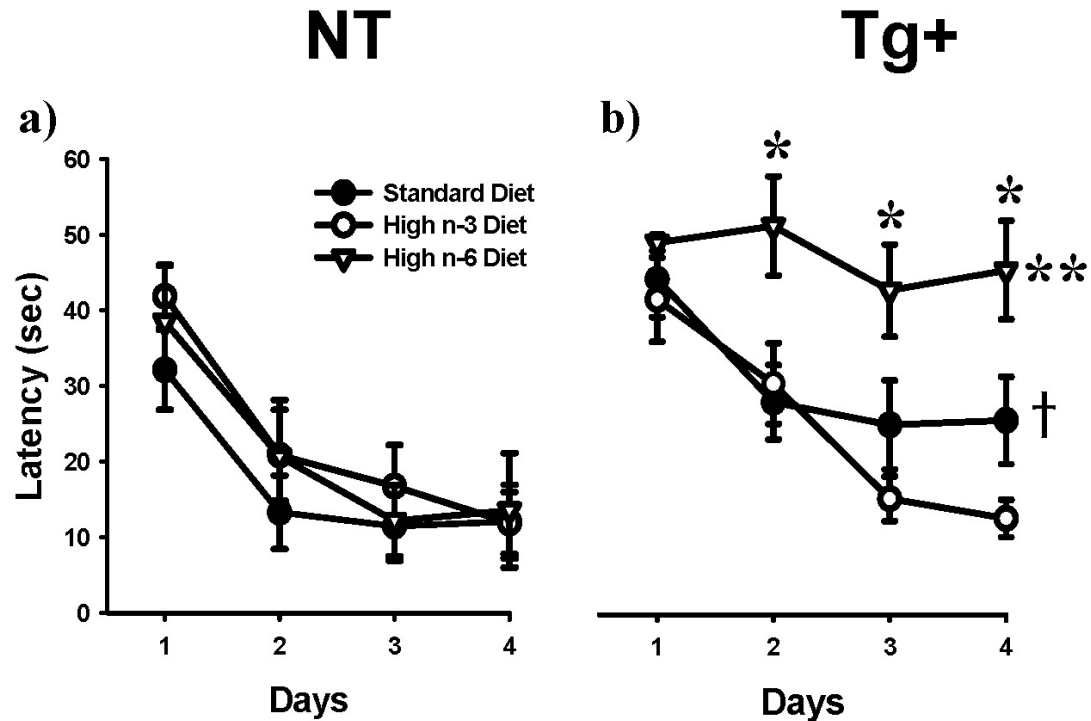


Fig. 30. Platform recognition testing for the ability to search/ identify a variably-placed and conspicuously marked platform over 4 days of testing, with latency to swim to the platform being measured. (a) There were no differences in latency between the diet groups of the NT mice, with all groups reducing their latency over days. (b) The Tg+ standard-fed mice, however, were impaired overall vs. NT standard-fed mice. In addition, the high n-6 Tg+ mice were significantly more impaired compared to the standard Tg+ mice. In sharp contrast, the high n-3 Tg+ mice performed significantly better compared to the high n-6 Tg+ groups overall and specifically during days 2 through 4 of testing, although their performance was not statistically better than that of standard Tg+ mice. † Significantly different from standard NT mice overall at $P < 0.05$, * significantly different from high n-3 Tg+ mice at $P < 0.02$ or higher level of significance, **significantly different from all other groups in overall performance at $P < 0.05$ or higher level of significance.

Platform Recognition

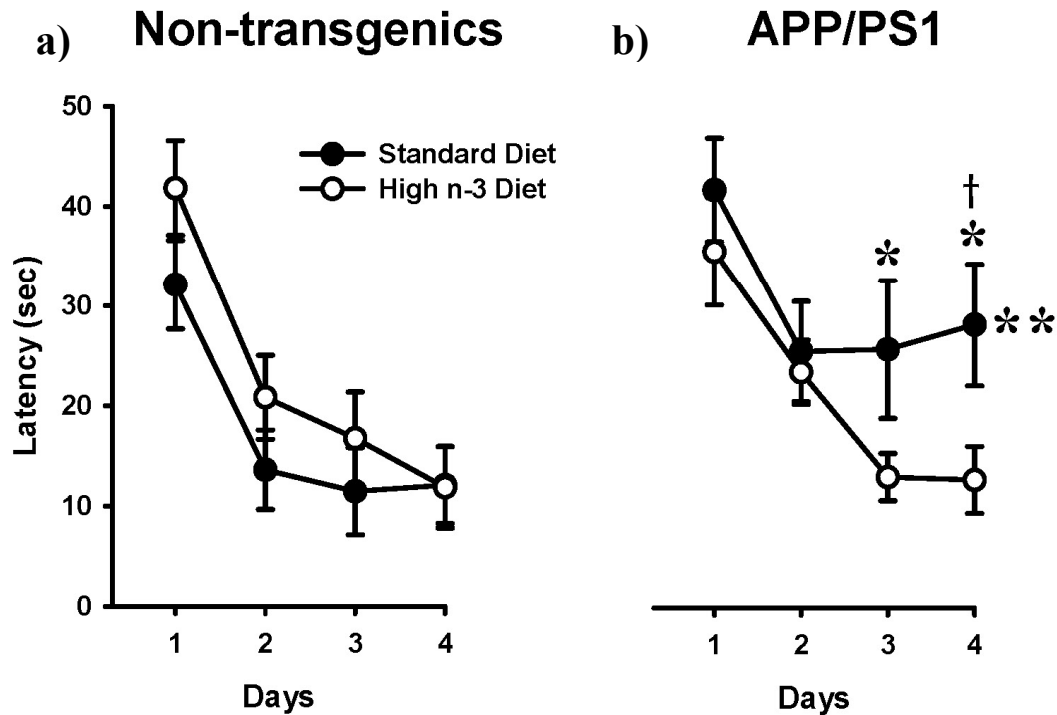


Fig. 31. Platform recognition, as assessed within NT and APP/PS1 standard and high n-3 diet groups. (a) There were no differences in latency between the diet groups of the NT mice, with both groups reducing their latency over days. (b) The Tg+ standard-fed mice, however, were impaired overall compared to standard NT mice. In sharp contrast, the high n-3 Tg+ mice performed significantly better compared to the standard APP/PS1 mice by day 4 of testing. † Significantly different from high n-3 APP/PS1 mice at $P<0.05$, * significantly different from standard NT mice at $P<0.05$ or higher level of significance, **significantly different from standard NT overall at $P<0.02$.

Radial Arm Water Maze. RAWM data are presented in terms of errors (Figures 32-34) and latency (Figures 35-37) to find the goal arm. All of the NT groups performed similar to each other across all three blocks (Fig. 32a) exhibiting no differences due to dietary intervention. In addition, all NT groups showed learning by exhibiting a significant reduction of errors from T1 to T4 and retaining that low number during T5 for both blocks 2 and 3 as well as overall three blocks. In contrast, the standard-fed Tg⁺ mice (Fig. 32b) had significantly more errors (were impaired) as compared to the standard-fed NT mice for T4 and T5 during block 2, and during T5 for the last block of testing. Within the Tg⁺ groups, there was no significant benefit of either experimental diet. However, the Tg⁺n-3 did make significantly more errors on block 1 T5 as compared to the standard Tg⁺ mice. With that exception, there were no other group differences within the Tg⁺ groups.

There were no differences in overall T4 or T5 errors within the NT groups (Fig. 33). Regarding Tg⁺ mice, only the standard Tg⁺ mice were impaired overall on T4 compared to the standard NT mice, while the high n-3 and high n-6 Tg⁺ performed similar to standard NT mice (Fig. 33a). More importantly, however, the overall performance of high n-3 and high n-6 Tg⁺ mice was not significantly better than standard Tg⁺ mice. Moreover, all 3 groups of Tg⁺ mice were equally impaired in overall T5 errors versus standard NT mice (Fig. 33b).

Comparison between only the standard and high n-3 dietary groups including only NT and APP/PS1 mice is presented as overall T4 and T5 errors in Figure 34. Regarding the NT mice, both groups performed similar to each other for both T4 and T5. Analysis pertaining solely to APP/PS1 mice showed that for both T4 and T5 overall errors, only

the standard APP/PS1 mice were impaired compared to the standard NT mice; performance of the high n-3 APP/PS1 mice was statistically not different from the same standard NT mice (Fig. 34). As was the case for all mice included (Figure 33), however, a high n-3 diet did not affect working memory (T4 and T5) performance of APP+PS1 mice compared to standard APP+PS1 mice. These data involving RAWM errors indicate that a diet high in n-3 and/or n-6 fatty acids has very minimal to no effect on working memory in Alzheimer's transgenic mice.

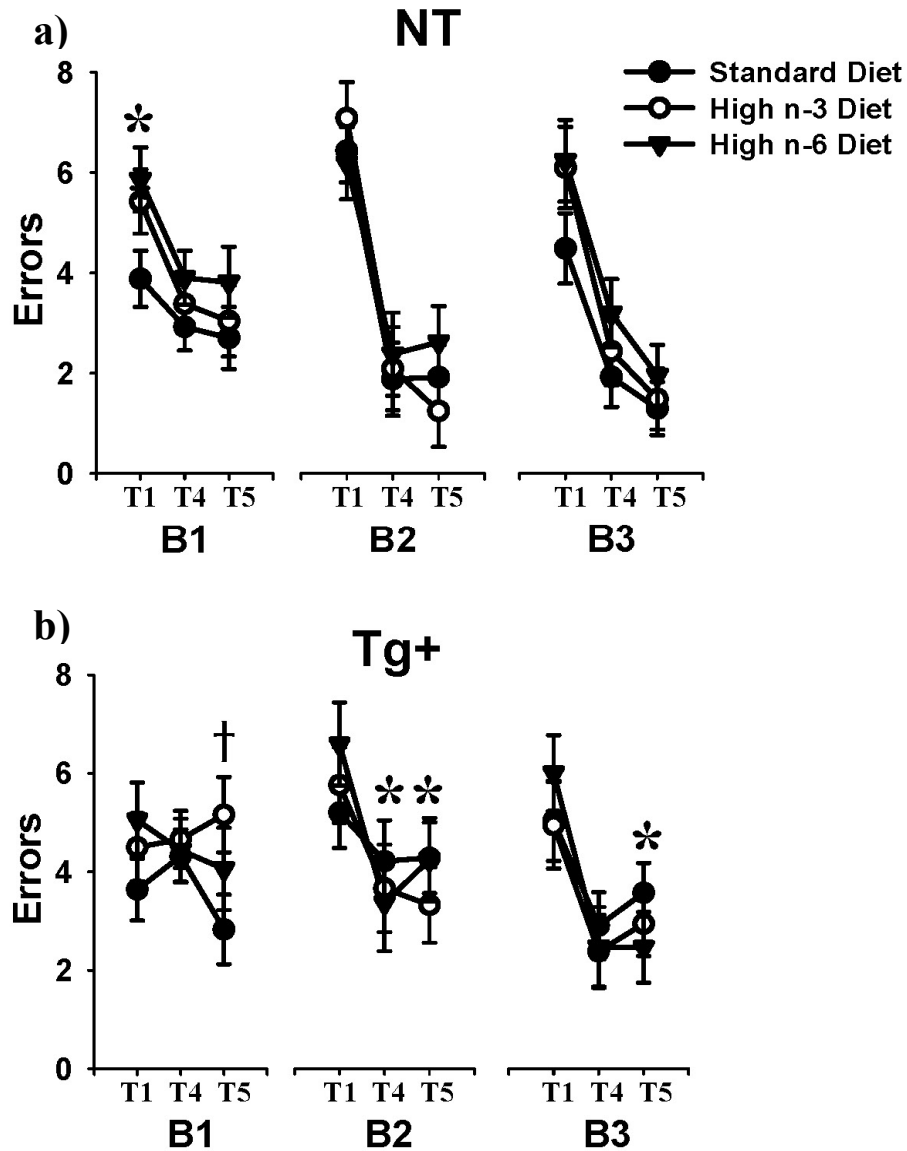


Fig 32. Working memory in the RAWM task in NT (a) and Tg+ (b) mice being evaluated for T1, T4 and T5 errors over three 3-day blocks. T4 and T5 are indices of working memory. All three NT groups performed similarly in being able to reduce their number of T4 and T5 errors across blocks. By contrast, standard Tg+ were impaired versus standard NT mice for T4 and T5 during block 2 and T5 only during block 3. Within the Tg+ groups, the n-3 mice were impaired as compared to standard Tg+ mice for T5 within block 1. Otherwise there were no differences due to diet within the Tg+ mice. *Standard Tg+ mice significantly different from standard NT mice at $P<0.05$ or higher level of significance. † High n-3 Tg+ significantly different from standard Tg+ mice at $P<0.05$.

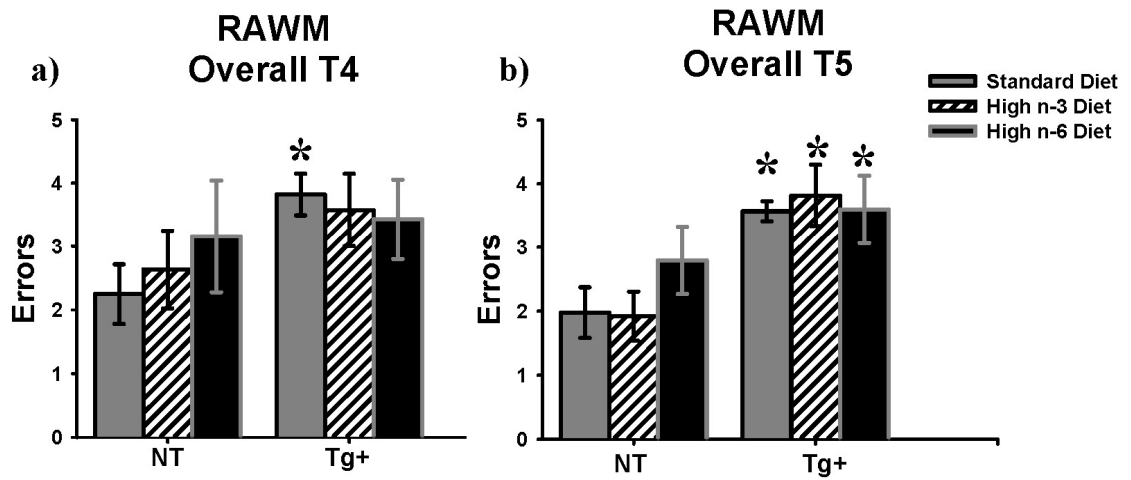


Fig 33. RAWM errors in overall T4 (a) and T5 (b) for both NT and Tg+ groups. All NT groups had similar overall performance in both T4 and T5. Although the standard Tg+ mice had increased errors in overall T4 compared to standard-fed NT mice, there were no differences in T4 performance between the three Tg+ groups. In overall T5 errors, all three Tg+ groups displayed similar working memory impairment in making an increased number of errors versus standard NT mice. *Significantly different from standard NT at $P < 0.05$.

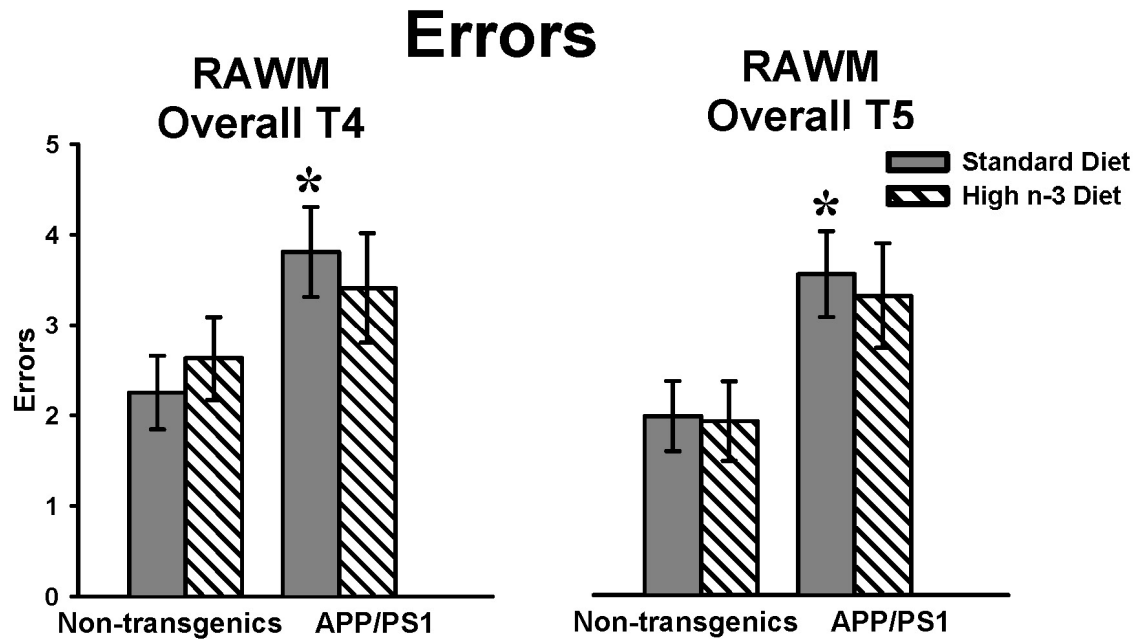


Fig 34. RAWM errors in overall T4 and T5 for both NT groups and APP/PS1 groups. All NT groups had similar overall performance in both T4 and T5. The standard APP/PS1 mice (but not the high n-3 mice) had increased T4 and T5 overall errors compared to standard-fed NT mice. However, the high n-3 diet had no effect on T4 and T5 working memory when the two APP+PS1 groups were compared directly to one another. *Significantly different from standard NT at $P<0.05$.

Latencies to find the hidden platform for T1, T4 and T5 across the three 3-day blocks of the RAWM task are graphed for both NT (Fig. 35a) and Tg+ (Fig. 35b) mice. Across all nine days of RAWM testing, there were no differences between any of the NT groups with respect to latency to find the hidden platform. As was the case for RAWM errors, by the final two blocks of testing (B2 and B3), all three NT groups showed excellent working memory, as evidenced by their low T4 and T5 latencies. By contrast, all three Tg+ groups had increased latencies for both working memory trials (T4 and T5) in blocks 1 and 2 compared to standard NT mice. This impairment in T4 and T5 performance continued into the final block for standard Tg+ mice vs. standard NT mice. Compared to standard Tg+ mice, high n-3 and high n-6 Tg+ mice had slightly lower T4 and T5 latencies in Block 3 (Fig. 35b), which resulted in both of these groups not being different from standard NT controls on those trials. However, in the more important direct comparison between the three Tg+ groups, no significant differences were noted in T4 and T5 performance during block 3 (or any other block). Underscoring the lack of a robust effect of high n-3 or high n-6 diets in Tg+ mice are results of overall T4 and T5 performance (Fig. 36). All three NT groups performed similar in showing excellent overall T4 and T5 performance (e.g., low latencies), while all three Tg+ groups showed similar overall T4 and T5 impairment compared to standard NT controls. Despite the somewhat improved performance of the high n-3 and high n-6 Tg+ groups by the last block, neither of these groups performed significantly better than standard-fed Tg+ controls for any RAWM latency measure and both groups were impaired by having higher overall latencies in T4 (Fig. 36a) and T5 (Fig. 36b) overall.

To determine any effects a high n-3 diet might have specifically on APP+PS1 mice, RAWM performance of only APP/PS1 mice was compared to NT controls in the last block of testing (Fig. 37). As with all other indexes of RAWM performance, a high n-3 diet did not improve the already excellent working memory performance of NT mice during this final block. However, standard Tg+ mice were unable to improve their performance between T1 and T4/5 while high n-3 mice were able to do so. Because of this better performance by high n-3 Tg+ mice, they were no different in T4 or T5 latencies compared to standard NT mice, while standard Tg+ mice were impaired on both T4 and T5 (Fig. 37). By T5 of the last block, there was a strong trend for the high n-3 APP/PS1 mice to have improved performance compared to the impairment of the standard APP/PS1 group ($P=0.06$). As with RAWM errors, the RAWM latency analysis suggests that dietary supplementation with n-3 or n-6 fatty acids results in slight or no improvement in working memory performance.

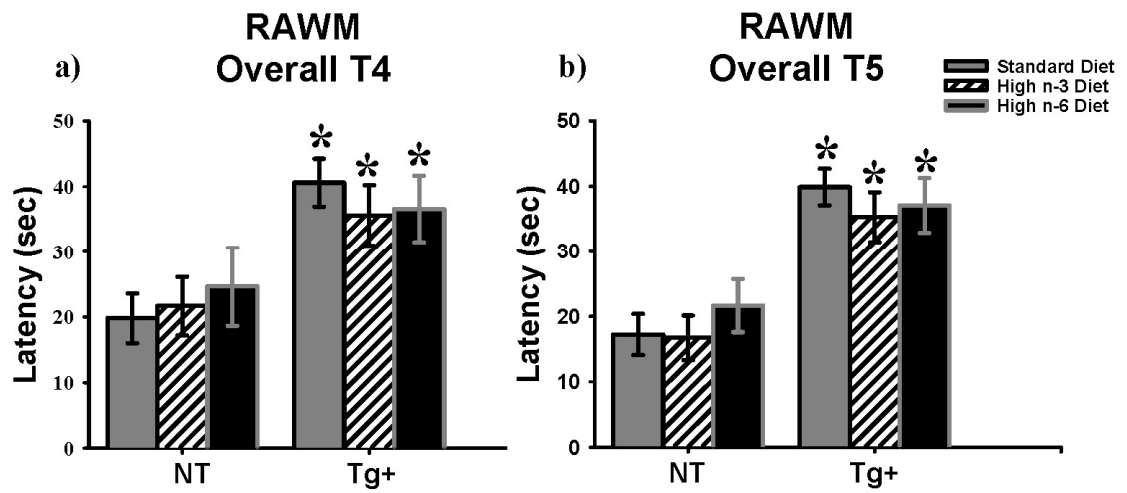


Fig 36. RAWM latency to find the hidden platform in overall T4 (a) and T5 (b) for both NT and Tg+ groups. All NT groups had similar overall performance in both T4 and T5, however, all three Tg+ groups had increased latencies in both T4 and T5 compared to the standard-fed NT mice. *Significantly different from standard NT at $P < 0.02$ or higher level of significance.

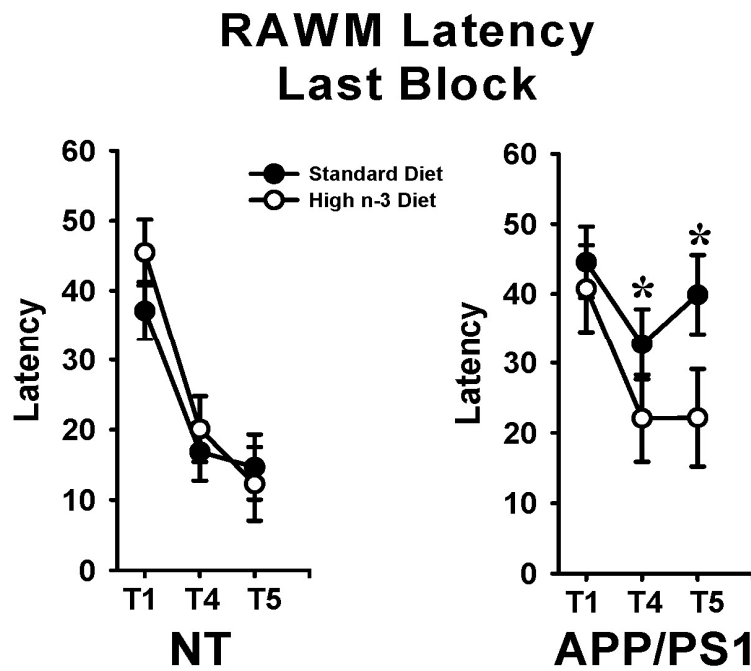


Fig 37. RAWM latency to find the hidden platform within the last block of testing for both NT and APP/PS1 on either the standard or high n-3 fatty acid diets. Both NT groups performed similar to each other and exhibited excellent learning by a decrease in latency from T1 to T4/T5. Likewise, the high n-3 APP/PS1 showed this learning effect, whereas the standard APP/PS1 could not improve their performance between T1 and T4/5. Also, the standard APP/PS1 had increased latencies versus standard NT mice for T4 and T5 (* $P < 0.05$ or greater level of significance vs. standard NT controls)

Cytokine Levels

At 7½-9 months of age (5½ -7 months into dietary treatments), pro-inflammatory and anti-inflammatory cytokines from plasma were measured in both NT (Fig. 38a) and APP/PS1 (Fig. 38b) mice from both the high n-3 and high n-6 diet groups. For NT mice, there were no differences in plasma levels of any cytokine for high n-3 vs. high n-6 diet groups (Fig. 38a). Among APP/PS1 mice, there was a consistent trend for high n-6 mice to have reduced levels of both pro- and anti-inflammatory cytokines compared to high n-3 mice (Fig. 38b). However, only levels of IL-1 β and IFN- γ were significantly reduced by a high n-6 diet vs. a high n-3 diet. Because there were no significant differences between the two genotypes for any cytokine irrespective of diet, cytokine levels from both genotypes were combined. As shown in Figure 39, the high n-6 diet mice (NT & APP/PS1) had consistently lower levels of all cytokines measured compared to all mice on the high n-3 diet. However, the only measure that was significantly reduced by the high n-6 diet was IL-1 β . Surprisingly, the anti-inflammatory cytokines, IL-4 and IL-10 were also slightly, but not significantly, lower in the high n-6 group. For both NT and APP/PS1 mice, these results indicate that the plasma cytokine profile is similar following a lengthy period of a high n-3 vs. a high n-6 diet.

Hippocampal A β levels

As measured by ELISA, there were no differences in levels of either soluble or insoluble A β within hippocampal tissue between any of the three APP/PS1 dietary groups (Fig. 40). Thus, neither a high n-3 or a high n-6 diet, administered over 5½ months, affected brain A β levels. There was however, a strong trend for the high n-6 APP/PS1

group to have a lower level of insoluble A β_{42} compared to both standard and high n-3 diet groups.

Non-transgenic

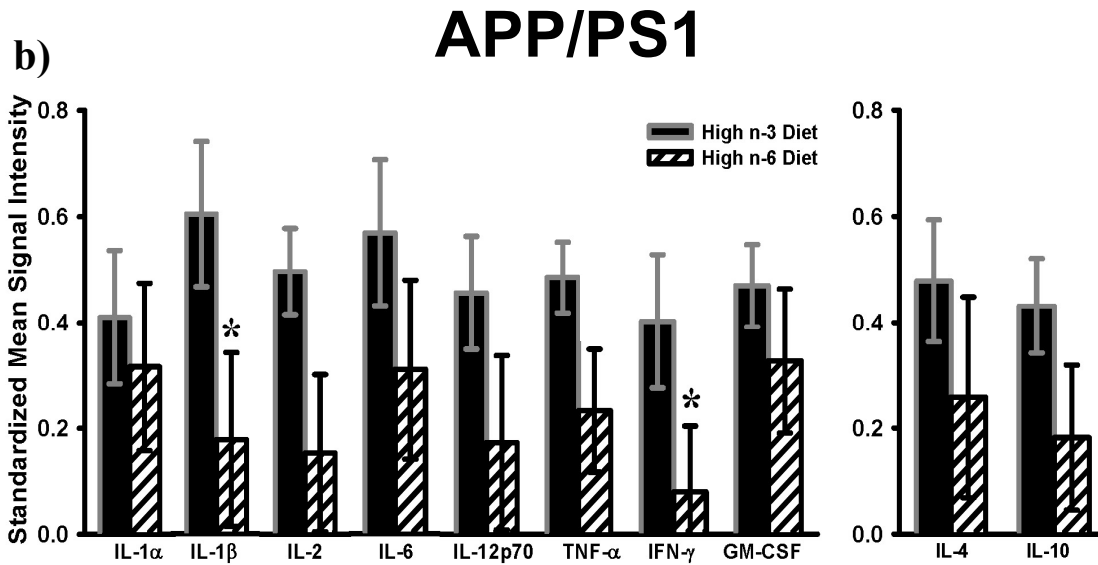
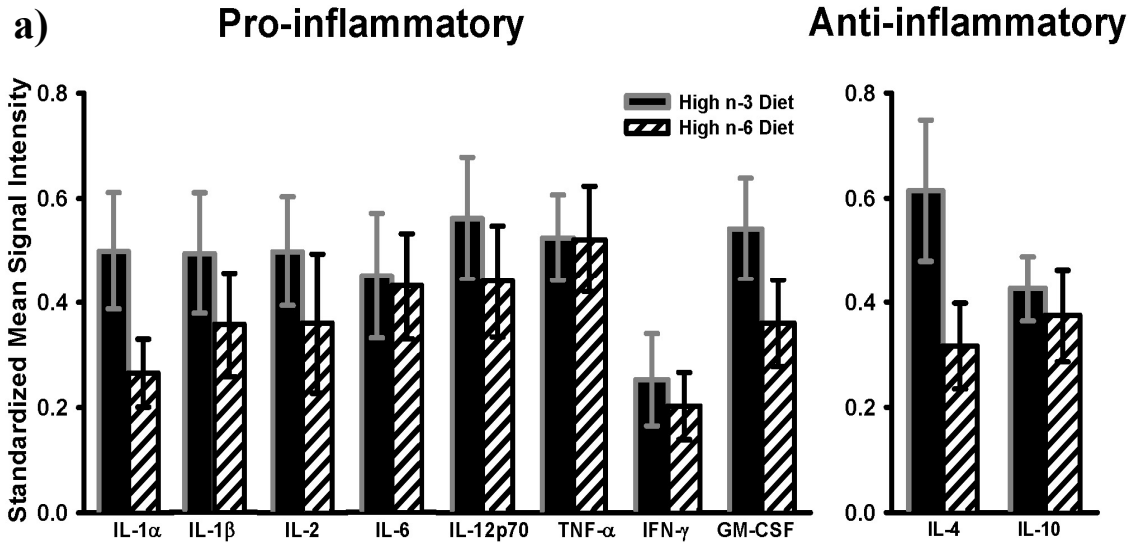


Fig. 38. Standardized mean signal intensity of plasma cytokines in both NT (a) and APP/PS1 (b) mice on either the high n-3 or high n-6 fatty acid diets. No differences were seen in any of the cytokine levels between the two NT groups for either pro- or anti-inflammatory cytokines. However, within the APP/PS1 groups, the high n-6 mice expressed lower levels of IL-1 β and IFN- γ compared to high n-3 APP/PS1 mice. Also, the high n-6 group had reduced expression of TNF- α compared to both NT groups. * Significantly different compared to high n-3 APP/PS1 mice at $P < 0.05$.

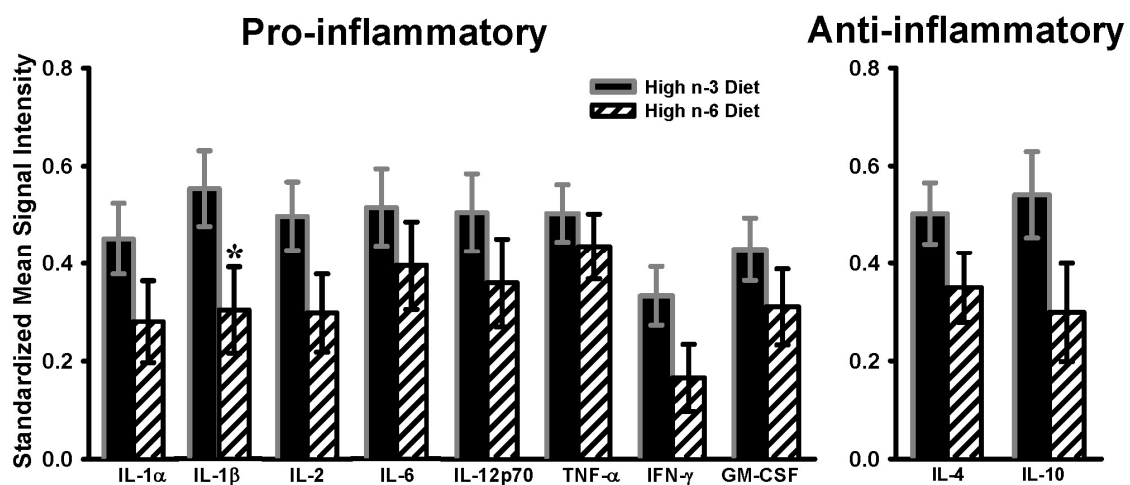


Fig. 39. Standardized mean signal intensity of plasma cytokines for all mice on either the high n-3 or high n-6 fatty acid diets irrespective of genotype. Although the high n-6 diet group had consistently lower levels across all cytokines, IL-1 β in high n-6 diet mice was the only cytokine that was significantly reduced vs. high n-3 mice. *Significantly different from high n-3 group at $P < 0.05$.

A β ELISA

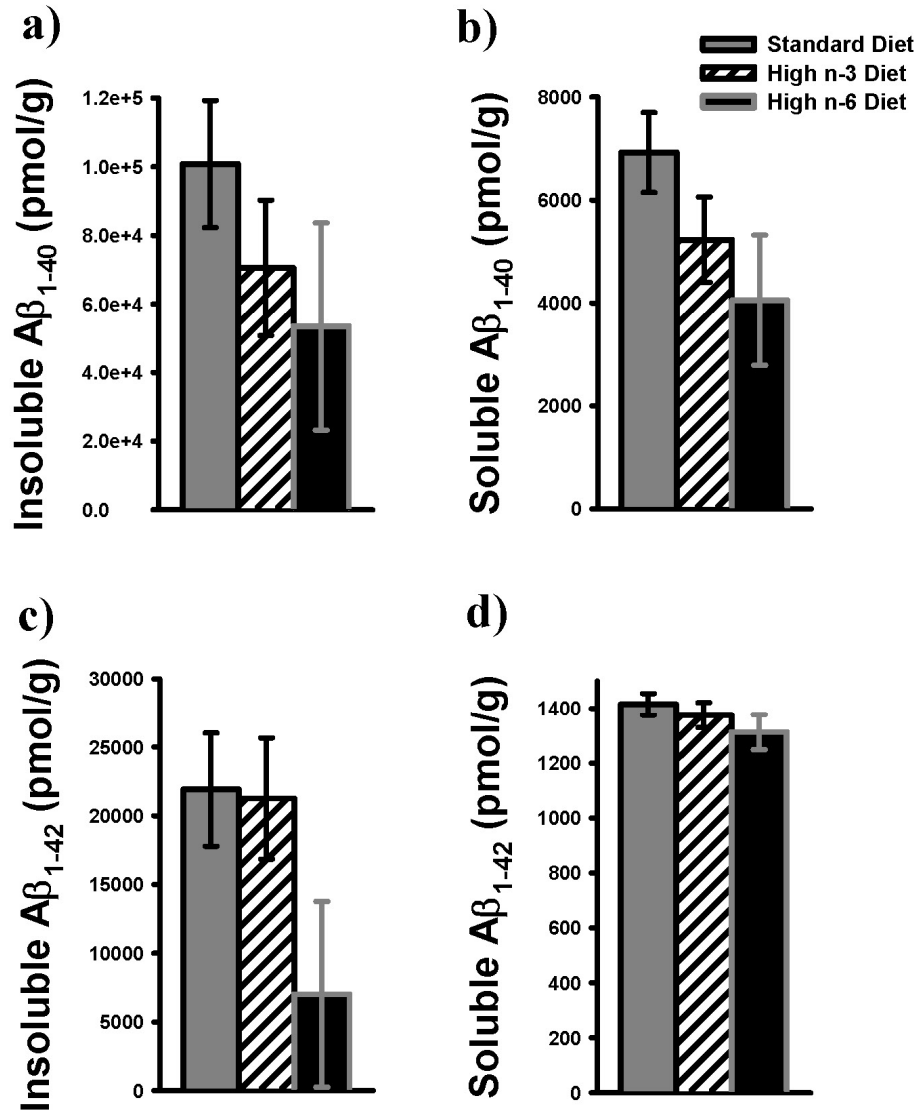
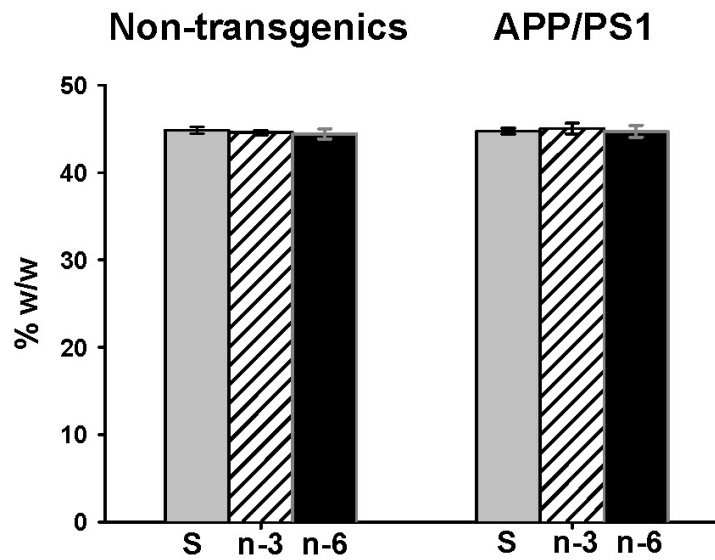


Fig. 40. Hippocampal A β levels (pmol/g), as measured by ELISA, of insoluble and soluble A β_{1-40} (**a**, **b**) as well as insoluble and soluble A β_{1-42} (**c**, **d**). No differences were found between any of the dietary groups for any A β marker.

Fatty Acid Brain Tissue Levels

Saturated and Mono-unsaturated Fatty Acids. Fatty acid levels including saturated, mono-unsaturated and polyunsaturated fatty acids were measured from frontal cortex and expressed as mean percentage of the total fatty acids. There were no differences in any of the saturated or mono-unsaturated fatty acids between any of the three NT groups (data not shown). Figure 41 illustrates that there were no differences in either total saturated (Fig. 41a) or total mono-unsaturated (Fig. 41b) fatty acids between the NT groups. Similarly, there were no differences in any of the saturated fatty acids within the three APP/PS1 groups or in their total saturated fatty acid level (Fig. 41a). There was also no difference in total mono-unsaturated fatty acids between any of the APP/PS1 groups (Fig. 41b).

a) Total Saturated Fatty Acids



b) Total Mono-unsaturated Fatty Acids

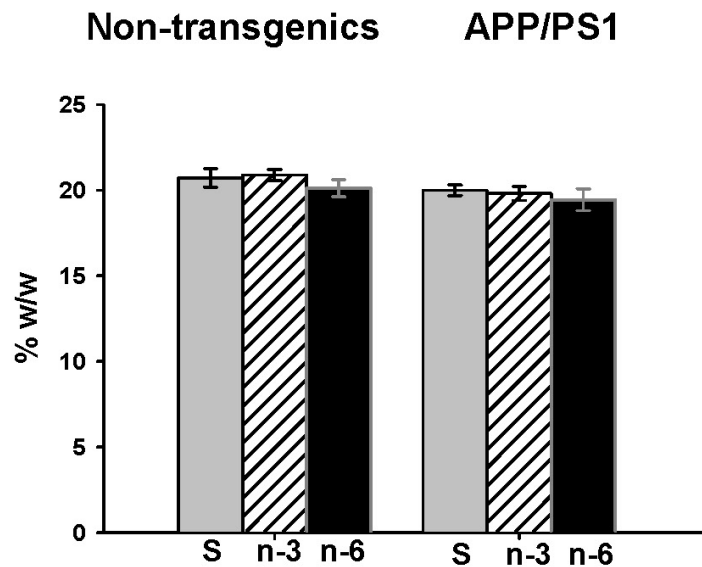


Fig. 41. Cortical levels of total saturated (a) and mono-unsaturated (b) fatty acid levels of standard, high n-3 and high n-6-fed NT and APP/PS1 mice. No differences were found between any of the groups for saturated or mono-unsaturated fatty acids. Values expressed as mean percentage of total fatty acids \pm SEM.

Omega-6 and Omega-3 Fatty Acids. As seen in Figure 42a for NT mice, the high n-6 group had elevated cortical levels of 18:2n-6 and 20:2n-6 as compared to both other groups. Likewise, the high n-3 mice had reduced levels of 20:4n-6 and 22:4n-6 as compared to both standard and high n-6 mice. Surprisingly, however, the high n-3 mice showed an elevation of one n-6 fatty acid, 20:3n-6. Despite this elevation, the high n-3 mice had reduced total n-6 fatty acids compared to both standard and high n-6 NT mice (Fig. 43a). Therefore, in the important direct comparison of the high n-3 diet versus the high n-6 diet, the high n-6-fed NT mice had increased levels of four of seven omega-6 fatty acids. Of the four different n-3's that were measured, 20:5n-3 and 22:5n-3 were both significantly elevated in the high n-3 NT mice compared to both other groups (Fig. 42a). Likewise, total n-3 fatty acids were higher in the n-3 fed NT group as compared to the standard-fed and high n-6 fed mice (Fig. 43b). Thus, in a direct comparison between the two experimental diets, the high n-3 diet resulted in increased omega-3 fatty acids within the frontal cortex in the NT mice.

Within the dietary groups of APP/PS1 mice, the results were less consistent. Figure 42b shows that only 22:5n-6 was significantly increased in the n-6 fed mice compared to the n-3 fed mice. However, reductions in 3 of the seven n-6 fatty acids (18:2n-6, 20:2n-6 and 20:3n-6) were found in the standard-fed mice compared to both experimental diet groups. As Figure 45a shows, there were no differences in total omega-6 fatty acids between any of the diet groups within the APP/PS1 mice. There were also no differences in any of the n-3 fatty acids measured in the APP/PS1 mice (Fig. 42b). Thus, the total n-3 fatty acids were similar between the three APP/PS1 diet groups, as seen in figure 43b.

Comparisons were also made within the individual diet groups to determine the effect of genotype on the fatty acid content in the frontal cortex (data not shown). For the standard-fed mice, there were no significant differences between any of the fatty acids measured for NT vs. APP/PS1 mice, except for elevations in both 22:5n-6 and 22:5n-3 in APP/PS1 mice. However, for the high n-3 diet mice, 5 of the seven n-6 fatty acids were elevated in APP/PS1 mice compared to NT mice (exceptions were 18:3n-6 and 20:3n-6), resulting in significantly higher total n-6 fatty acid levels in cortex of APP/PS1 mice vs. NT controls. In addition, 20:5n-3 and 22:5n-3 were reduced in the APP/PS1 mice versus NT mice on the high n-3 diet. The reduction in these two n-3 fatty acids resulted in a significant reduction in total n-3 fatty acids in the APP/PS1 mice. Lastly, for mice fed the high n-6 diet, only 20:2n-6 was elevated in APP/PS1 mice compared to NT mice. Thus, for mice fed the high n-6 diet, there were no genotypic differences in any of the other six n-6 fatty acids, or any genotypic differences for any of the n-3 fatty acids.

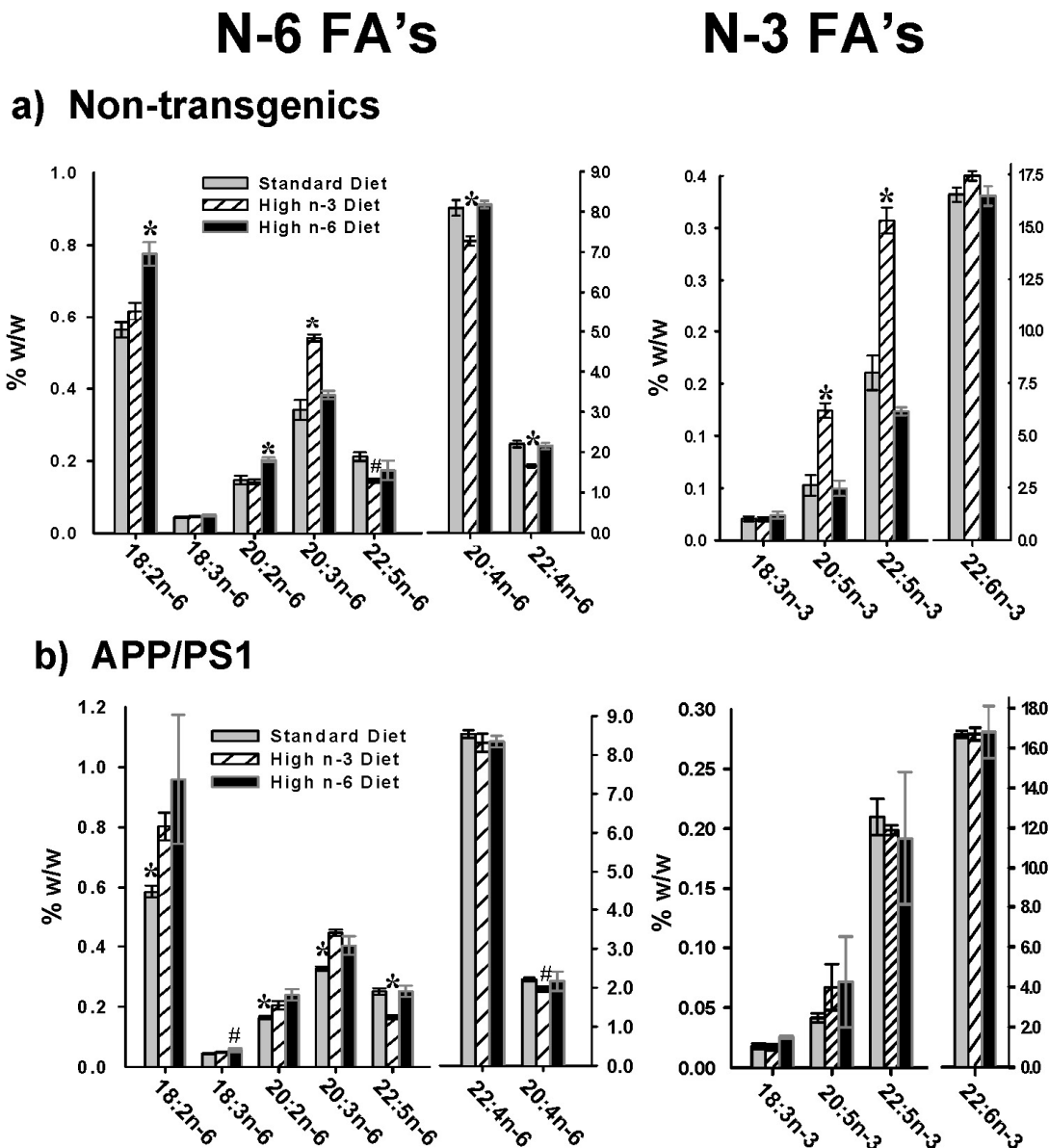


Fig. 42. Cortical levels of n-6 and n-3 fatty acids for standard, high n-3 and high n-6 NT (a) and APP/PS1 (b) mice. Within the NT mice, the high n-6 diet group showed increases in four of seven n-6 fatty acids compared to the high n-3 diet group. Conversely, high n-3 diet mice had elevations in two of four omega-3 fatty acids, 20:5n-3 and 22:5n-3, as compared to both other NT groups. Within the APP/PS1 mice, the high n-6 group had elevations in only one n-6 fatty acid (22:5n-6) versus the high n-3 mice. However, the standard-fed mice had lower levels of three n-6 fatty acids compared to both other groups. There were no differences in omega-3 fatty acids between any of the three APP/PS1 groups. Values expressed as mean percentage of total fatty acids \pm SEM. *Significantly different from both other diet groups within that genotype at $P < 0.05$ or greater level of significance. # Significantly different from standard-fed group within that genotype at $P < 0.05$ or higher level of significance.

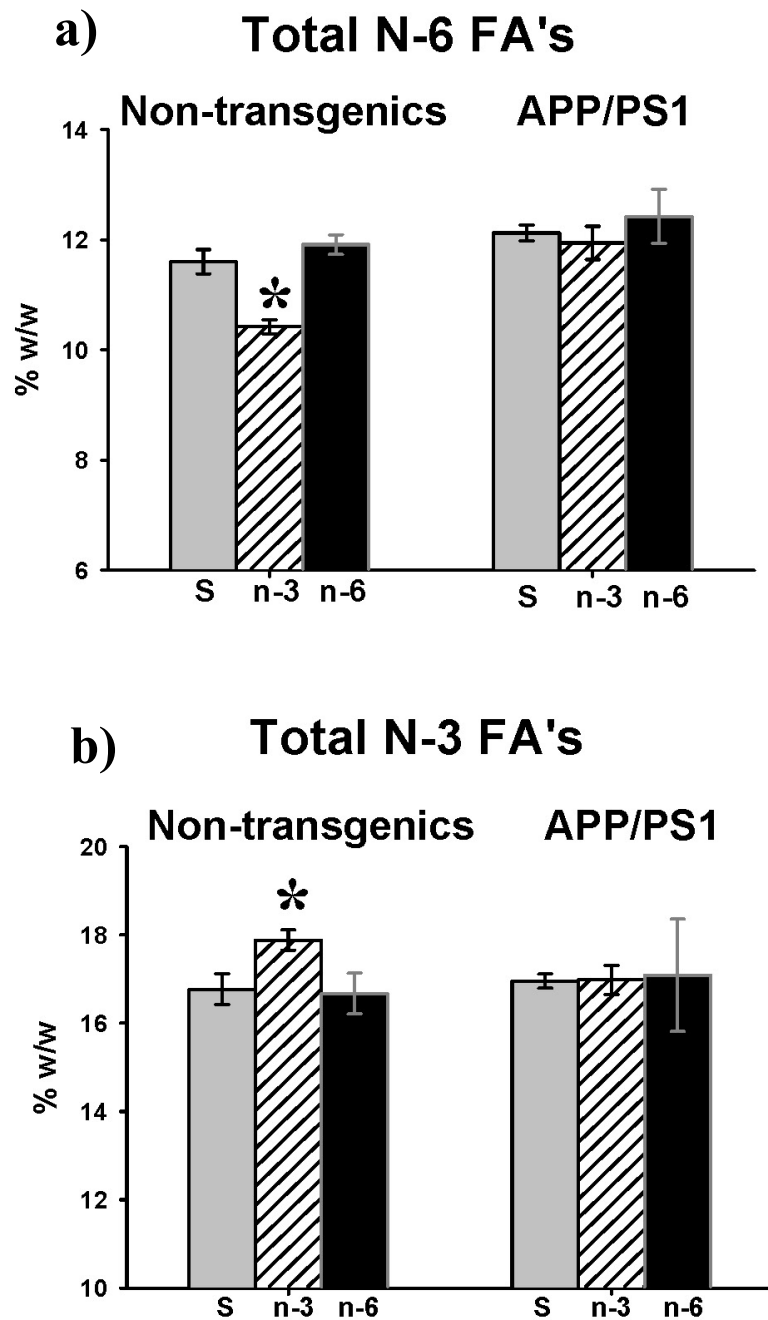


Fig. 43. Total n-6 (a) and total n-3 (b) fatty acid levels in standard, high n-3 and high n-6 NT and APP/PS1 mice. High n-3 NT mice had significantly lower total n-6 as compared to both other NT groups. Similarly, high n-3 NT mice had significantly elevated n-3 as compared to both standard and high n-6-fed mice. There were no differences between the APP/PS1 groups for either total n-6 or n-3 fatty acid levels in frontal cortex. Values expressed as mean percentage of total fatty acids \pm SEM. * Significantly different from both standard-fed and high n-6-fed NT groups at $P < 0.05$ or higher level of significance.

Correlations and Multimetric Analyses

Correlations were performed involving both NT and APP/PS1 mice for behavioral measures versus fatty acid levels (saturated, mono-unsaturated and poly-unsaturated) from the frontal cortex, plasma cytokines, and hippocampal A β levels. First, behavioral measures of NT mice, combining all three diet groups, were correlated with cortical fatty acid levels. Then, NT mice from only the high n-3 and high n-6 groups were used to correlate behavior with both fatty acid levels and plasma cytokines. Because all pathology factors were only measured in APP/PS1 mice, a final two sets of correlations excluded all APP mice and therefore also excluded behavioral measures from the high n-6 mice. Therefore, correlations were performed between standard and high n-3 APP/PS1 mice behavioral measures and both cortical fatty acids and hippocampal A β levels. Lastly, A β levels, cytokines and fatty acid levels were correlated within the high n-3 and high n-6 APP/PS1 mice.

Correlations involving all NT groups. Significant correlations involving all NT mice combined from the three diet groups revealed associations between n-6 or n-3 fatty acids in the frontal cortex with cognitive impairment (Table 10). More specifically, 20:2n-6 (di-homo linoleic acid) was negatively correlated with Morris water maze retention, such that an increased amount of this fatty acid in the brain was associated with less time spent in the former platform-containing quadrant during the probe trial. In addition, levels of this same fatty acid as well as 22:5n-6 (docosapentaenoic acid) were correlated with increased latency to find the hidden platform in RAWM. Surprisingly, 22:6n-3 (DHA) and total N-3 PUFAs were positively correlated to impairment in both Morris water maze acquisition and RAWM.

Correlations involving high n-3 and high n-6 NT groups. Additional correlations, including only the high n-3 and high n-6 fatty acid diets were also performed with the NT mice involving behavioral performance, brain fatty acid levels, and plasma cytokines (Table 10). Many significant correlations arose between impairment in platform recognition and all seven n-6 fatty acids, as well as 2 of 4 n-3 fatty acids, indicating a clear association between high brain lipid levels and impaired search/recognition and identification learning. Despite no differences between the n-3 and n-6 NT dietary groups in saturated or mono-unsaturated fatty acid levels, there were significant and consistent associations between high levels of eight out of ten saturated fatty acids and impairment in the platform recognition task for both NT groups combined. Also, six out of ten mono-unsaturated fatty acid levels were significantly correlated to impairment in the same task for both n-3 and n-6 NT groups combined. In addition, all n-6 fatty acids measured and two of four n-3 fatty acids were significantly correlated with impairment in the platform recognition task for both n-3 and n-6 NT groups combined. There were essentially no correlations between plasma cytokine levels and cognitive performance for the combined high n-3 and high n-6 NT diet groups.

Table 10. Correlations for all NT mice combined between cortical fatty acid levels and behavioral measures or for only high n-3 and high n-6 NT mice between fatty acid levels, plasma cytokines and behavioral performance. All abbreviations defined below in Table 12.

| All NT mice | r value | p value |
|---|----------------|----------------|
| WM-Ret vs. 20:2n-6 | -0.533 | 0.007 |
| RAWM-T4-B3 vs. 20:2n-6 | 0.440 | 0.026 |
| RAWM-T5-B3 vs. 20:2n-6 | 0.399 | 0.036 |
| RAWM-T5-B3 vs. 22:5n-6 | -0.434 | 0.039 |
| WM-Fin vs. 22:6n-3 | 0.442 | 0.030 |
| RAWM-T5 vs. 22:6n-3 | 0.414 | 0.050 |
| RAWM-T5-B3 vs. 22:6n-3 | 0.489 | 0.018 |
| WM-Fin vs. Totaln-3 | 0.448 | 0.028 |
| RAWM-T5-B3 vs. Totaln-3 | 0.457 | 0.028 |
| | | |
| High n-3 and high n-6 diet NT mice | r value | p value |
| PR-Avg vs. 12:0 | 0.652 | 0.008 |
| PR-Avg vs. 16:0DMA | 0.618 | 0.014 |
| PR-Fin vs. 16:0DMA | 0.743 | 0.001 |
| PR-Avg vs. 16:0 | 0.544 | 0.036 |
| PR-Fin vs. 16:0 | 0.652 | 0.008 |
| PR-Avg vs. 18:0DMA | 0.620 | 0.014 |
| PR-Fin vs. 18:0DMA | 0.743 | 0.002 |
| PR-Avg vs. 18:0 | 0.553 | 0.032 |
| PR-Fin vs. 18:0 | 0.657 | 0.008 |
| PR-Avg vs. 20:0 | 0.529 | 0.042 |
| PR-Fin vs. 20:0 | 0.675 | 0.006 |
| PR-Fin vs. 22:0 | 0.634 | 0.011 |
| PR-Fin vs. 24:0 | 0.666 | 0.007 |
| PR-Fin vs. 16:1n-7 | 0.240 | 0.009 |
| PR-Avg vs. 18:1DMA | 0.616 | 0.014 |
| PR-Fin vs. 18:1DMA | 0.743 | 0.001 |
| PR-Avg vs. 18:1n-9 | 0.565 | 0.028 |
| PR-Fin vs. 18:1n-9 | 0.705 | 0.003 |
| PR-Avg vs. 20:1n-9 | 0.582 | 0.023 |
| PR-Fin vs. 20:1n-9 | 0.719 | 0.003 |
| PR-Avg vs. 22:1n-9 | 0.532 | 0.041 |
| PR-Fin vs. 22:1n-9 | 0.682 | 0.005 |
| PR-Fin vs. 24:1n-9 | 0.637 | 0.011 |
| PR-Fin vs. 18:2n-6 | 0.650 | 0.009 |
| PR-Avg vs. 18:3n-6 | 0.540 | 0.038 |
| PR-Fin vs. 18:3n-6 | 0.667 | 0.007 |
| PR-Avg vs. 20:2n-6 | 0.573 | 0.026 |
| PR-Fin vs. 20:2n-6 | 0.679 | 0.005 |
| PR-Avg vs. 20:3n-6 | 0.602 | 0.018 |
| PR-Fin vs. 20:3n-6 | 0.620 | 0.014 |
| PR-Fin vs. 20:4n-6 | 0.644 | 0.010 |
| PR-Fin vs. 22:4n-6 | 0.638 | 0.011 |
| PR-Fin vs. 22:5n-6 | 0.642 | 0.010 |
| PR-Avg vs. 18:3n-3 | 0.563 | 0.029 |
| PR-Fin vs. 18:3n-3 | 0.680 | 0.005 |
| PR-Avg vs. 22:6n-3 | 0.584 | 0.022 |
| PR-Fin vs. 22:6n-3 | 0.658 | 0.008 |

Correlations involving standard and high n-3 APP/PS1 groups. Significant correlations were found between combined standard-fed and high n-3-fed APP/PS1 groups versus behavioral measures, brain fatty acid, and A β levels (Table 11). Most notably, elevated hippocampal soluble A β 40 was significantly associated with Morris water maze acquisition and retention impairment, in addition to increased latencies for platform recognition and RAWM. As well, insoluble A β 40 also significantly correlated with impairments in Morris water maze and platform recognition. Soluble and insoluble A β 42 were not as well correlated with cognitive measures, although high levels of A β 42 were associated with poorer RAWM performance.

In addition to A β levels, increased levels of several cortical n-6 fatty acids also correlated with cognitive deficits in Morris water maze acquisition and retention, as well as cognitive impairment in RAWM. Surprisingly, cortical levels of n-3 fatty acids were essentially not associated with improvement in any of the cognitive-based measures.

Correlations involving high n-3 and high n-6 APP/PS1 groups. Significant correlations were found between hippocampal A β levels, plasma cytokines and cortical fatty acid levels in high n-3 and high n-6 APP/PS1 mice (Table 11). There were significant positive correlations between insoluble A β 42 and plasma cytokines, IFN- γ and IL-10, indicating both pro-inflammatory and anti-inflammatory associations with A β . However, 20:2n-6, 20:4n-6 and 22:5n-6 all negatively correlated with pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-2 or IL-6. In addition, the n-3 fatty acid, 18:3n-3 (ALA) also negatively correlated with many of the pro-inflammatory cytokines as well as with the anti-inflammatory cytokine, IL-10. Together, these correlations indicate that

increased cortical levels of n-6 and n-3 fatty acids are associated with an overall anti-inflammatory effect.

Table 11. Correlations for combined standard and high n-3 APP/PS1 mice between behavioral measures, cortical fatty acid levels and hippocampal A β levels or for combined high n-3 and high n-6 APP/PS1 mice between hippocampal A β levels, plasma cytokines and brain fatty acid levels. * InsolA β 42 = insoluble A β 42 within hippocampus; all other abbreviations defined below in Table12.

| Standard and High n-3 APP/PS1 mice | r value | p value |
|---|----------------|----------------|
| WM-Fin vs. SolA β 40 | 0.647 | 0.023 |
| WM-Ret vs. SolA β 40 | 0.490 | 0.005 |
| PR-Fin vs. SolA β 40 | 0.619 | 0.032 |
| RAWM-T5 vs. SolA β 40 | 0.759 | 0.011 |
| RAWM-T4-B3 vs. SolA β 40 | 0.754 | 0.012 |
| RAWM-T5-B3 vs. SolA β 40 | 0.896 | 0.001 |
| WM-Fin vs. InsolA β 40 | 0.519 | 0.005 |
| PR-Fin vs. InsolA β 40 | -0.422 | 0.040 |
| RAWM-T4 vs. InsolA β 42 | 0.706 | 0.023 |
| WM-Avg vs. 20:4n-6 | 0.855 | 0.001 |
| WM-Fin vs. 20:4n-6 | 0.733 | 0.007 |
| WM-Ret vs. 20:4n-6 | -0.786 | 0.002 |
| RAWM-T4-B3 vs. 20:4n-6 | 0.650 | 0.042 |
| RAWM-T5-B3 vs. 20:4n-6 | 0.695 | 0.026 |
| WM-Fin vs. 22:4n-6 | 0.610 | 0.035 |
| RAWM-T5-B3 vs. 22:4n-6 | 0.669 | 0.034 |
| WM-Avg vs. 22:5n-6 | 0.608 | 0.036 |
| PR-Fin vs. 22:5n-6 | 0.736 | 0.006 |
| RAWM-T5-B3 vs. 22:5n-6 | 0.855 | 0.002 |
| | | |
| High n-3 and high n-6 APP/PS1 mice | r value | p value |
| InsolA β 42 vs. IFN- γ | 0.718 | 0.019 |
| InsolA β 42 vs. IL-10 | 0.636 | 0.048 |
| 20:2n-6 vs. IFN- γ | -0.696 | 0.025 |
| 20:4n-6 vs. IL-6 | -0.658 | 0.039 |
| 22:5n-6 vs. IL-2 | -0.656 | 0.039 |
| 22:5n-6 vs. TNF- α | -0.634 | 0.049 |
| 18:3n-3 vs. GM-CSF | -0.662 | 0.037 |
| 18:3n-3 vs. IL-12p70 | -0.661 | 0.037 |
| 18:3n-3 vs. IFN- γ | -0.818 | 0.004 |
| 18:3n-3 vs. IL-10 | -0.678 | 0.031 |
| 18:3n-3 vs. IL-1 β | -0.668 | 0.035 |
| 18:3n-3 vs. TNF- α | -0.645 | 0.044 |

Table 12 shows the factor analysis of behavioral measures including all animals in all 6 treatment/genotype groups on a standard, high n-3 or high n-6 fatty acid diet. The table also includes a factor analysis for only the NT and APP/PS1 mice on either the standard or high n-3 diet, with and without pathologic measures. Including all 6 experimental groups, 16 of the 19 behavioral measures analyzed loaded on four principle factors, which together account for more than 65% of the total variance. All measures of RAWM errors and platform recognition, plus two measures of Morris water maze, loaded heavily in Factor 1, which was clearly cognitively-based and accounted for 33% of the total variance. In contrast, Factor 2 was strongly sensorimotor/anxiety based, including both elevated plus maze and balance beam measures. Factor 3 included both circular platform measures as well as Y-maze activity, while Factor 4 contained a single measure - the number of closed arm choices in elevated plus maze testing. Utilizing only two diet groups (standard and high n-3) and including only NT and APP/PS1 animals, many of the above 19 behavioral measures loaded similarly in Factors 1 and 2 (Table 10). Factor 1 was again cognitively- based in loading all measures of RAWM errors, platform recognition, and all three measures of Morris water maze. This factor, therefore, encompassed working memory, reference learning/ memory and recognition/ identification. Factor 2 was anxiety-based in loading only measures of elevated plus maze, while Factor 3 loaded two measures of activity/exploratory behavior.

Including neurochemical measures of cortical fatty acids (total saturated, total mono-unsaturated, total n-3, total n-6, and individual n-3 and n-6 fatty acids), as well as hippocampal soluble and insoluble A β levels to the behavioral measures for the NT and APP/PS1 groups is also reported in Table 12. Loading on Factor 1 were 10 cognitive

measures, soluble A β 40 and A β 42, and four n-6 fatty acid measures (including total n-6 fatty acid levels). Factor 1 contributed over 31% to the total variance. Factor 2 contained only fatty acid measures - two individual n-6 fatty acids and three individual n-3 fatty acids. Factor 3 contained the same measures of elevated plus maze as Factor 2 without the pathology measures. In addition, string agility and two measures of n-3 fatty acids also loaded on Factor 2. Lastly, total mono-unsaturated fatty acids loaded separately on Factor 4. In summary, factor loadings with and without pathologic measures were similar for Factor 1, though clearly there was an association between these measures and both A β pathology and cortical fatty acid levels.

Table 12. Factor loadings of behavioral measures, with and without pathologic measures.

| Factor | NT & Tg+ 6 Groups | NT & APP/PS1 4 Groups | NT & APP/PS1 4 Groups with Pathology |
|--------|--|--|---|
| 1 | (33.54) RM-T4-B3 RM-T5-B3 RM-T4 RM-T5 WM-Avg WM-Fin PR-Avg PR-Fin | (36.83) RM-T4-B3 RM-T5-B3 RM-T4 RM-T5 WM-Avg WM-Fin WM-Ret PR-Avg PR-Fin | (31.34) RM-T4-B3 RM-T5-B3 RM-T5 WM-Avg WM-Fin WM-Ret PR-Avg PR-Fin CPE-Avg YM-Alt SolA β 40 SolA β 42 20:4n-6 22:5n-6 22:4n-6 Totaln-6 |
| 2 | (14.69) WM-Ret EP-#O EP-TO BB | (15.08) EP-#O EP-TO | (17.29) 20:2n-6 18:3n-6 20:5n-3 18:3n-3 18:2n-3 |
| 3 | (12.46) CPE-Avg CPE-Fin YM-Ent | (11.3) YM-Ent EP-#C | (15.83) EP-TO EP-#O STR 22:6n-3 Totaln-3 |
| 4 | (8.09) EP-#C | (8.92) CPE-Avg | (10.31) TotMono |

Note: Percent of total variance explained by a given factor is indicated in bold type within parentheses.

Measures from the same task or pathologic markers are color-coded to facilitate identification.

BB = balance beam; EP-#C = # closed arm entries in elevated plus maze; EP-#O = # open arm entries in elevated plus maze; EP-TO = % time in open arm in elevated plus maze; PR-Avg = platform recognition overall latency; PR-Fin = platform recognition final day latency; RM-T4 = radial arm water maze errors overall trial 4; RM-T5 = radial arm water maze errors overall trial 5; RM-T4-Fin = radial arm water maze errors last block trial 4; RM-T5-Fin = radial arm water maze errors last block trial 5; SolA β 40 = soluble A β 40 within hippocampus; SolA β 42 = soluble A β 42 within hippocampus; TotMono = total mono-unsaturated fatty acids within frontal cortex; Totaln-3 = total omega-3 fatty acids within frontal cortex; Totaln-6 = total omega-6 fatty acids within frontal cortex; WM-Avg = water maze overall latency; WM-Fin = water maze latency last day; WM-Ret = water maze % time spent in Q2 during probe trial; YM-Ent = Y-maze arm entries; Individual n-3 and n-6 fatty acids are identified by ending in either n-3 or n-6

Discriminant function analysis (DFA) was used in order to determine if the overall behavioral performance of each of the three diet groups (standard, high n-3 and high n-6) could be distinguished from each other for both NT and Tg+ groups. Table 11 shows a summary of two DFA methods: the “direct entry” method (which included all 19 behavioral measures) and the “step-wise forward” method (which selected behavioral measures from all the measures based on their contribution to the variance). The direct entry DFA could not distinguish between the three groups for either NT or Tg+ mice based on their overall behavioral performance. However, the step-wise forward DFA, iteratively selecting from the 19 behavioral measures, could distinguish between the NT high n-6 group and both other NT groups. This discrimination was based on a single behavioral measure - circular platform errors on the final day of testing, wherein high n-6 NT mice were markedly impaired vs. the other two groups.

Within the Tg+ groups, the step-wise forward DFA was successful in distinguishing between all three diet groups so that the percent of animals correctly classified was 100% according to the classification matrix. Nine of the 19 behavioral measures were retained as providing the highest level of discriminability within the Tg+ groups: 8 cognitive measures and 1 anxiety/activity measure. A Canonical plot of these stepwise-forward DFA results involving Tg+ mice is presented in Figure 44. Similar discrimination was seen when including only APP/PS1 mice and comparing standard and high n-3 dietary groups (Table 12). A step-wise forward DFA using all behavioral measures could successfully discriminate between both standard and high n-3 APP/PS1 groups. Similar to the DFA that utilized all three Tg+ groups, the percent of animals correctly classified by the step-wise forward DFA including only APP/PS1 animals was

100%. Although both step-wise forward DFA's did provide discrimination between Tg+ groups behaviorally, no group was clearly superior across all of the behavioral measures iteratively selected therein. Moreover, direct entry DFA's were completely unsuccessful in distinguishing overall behavioral performance of the three Tg+ groups. Thus, there was no overall benefit or impairment provided by either the n-3 or n-6 diets in Tg+ mice.

Four additional DFAs were performed, which included only the eight cognitive-based measures that loaded into Factor 1 from Table 10. For the NT mice, neither the direct entry nor stepwise-forward DFAs could distinguish between any of the three dietary groups (Fig. 45a). Likewise, the direct entry DFA method could not discriminate between any of the Tg+ groups (Fig. 45b). However, a stepwise-forward DFA, selecting from the 8 cognitive-based measures from Factor 1 revealed discrimination between Tg+ high n-6 mice and both Tg+ standard and Tg+ high n-3 mice. The measures retained as providing maximal discrimination were platform recognition on the last day of testing (a search recognition/identification-based measure) and T5 latency during the last block of RAWM testing. When repeating the above DFA's for only APP/PS1 mice and only for standard diet vs. high n-3 diet, the two APP/PS1 groups could be distinguished when a stepwise-forward DFA was utilized and only Factor 1 cognitive-based measures were included. The two measures retained as providing this discrimination were RAWM last block T5 and RAWM overall T5 latency. For both of these cognitive measures, high n-3 mice had better performance. The percent of animals correctly classified by the step-wise forward DFA for Factor 1 for all Tg+ animals and only APP/PS1 animals was 72% and 90%, respectively. Nonetheless, for all three other DFA's that were done involving APP/PS1 mice alone, there was no discrimination between high n-3 and standard diet

groups, again underscoring that a high n-3 diet did not provide substantially better overall performance across a variety of tasks.

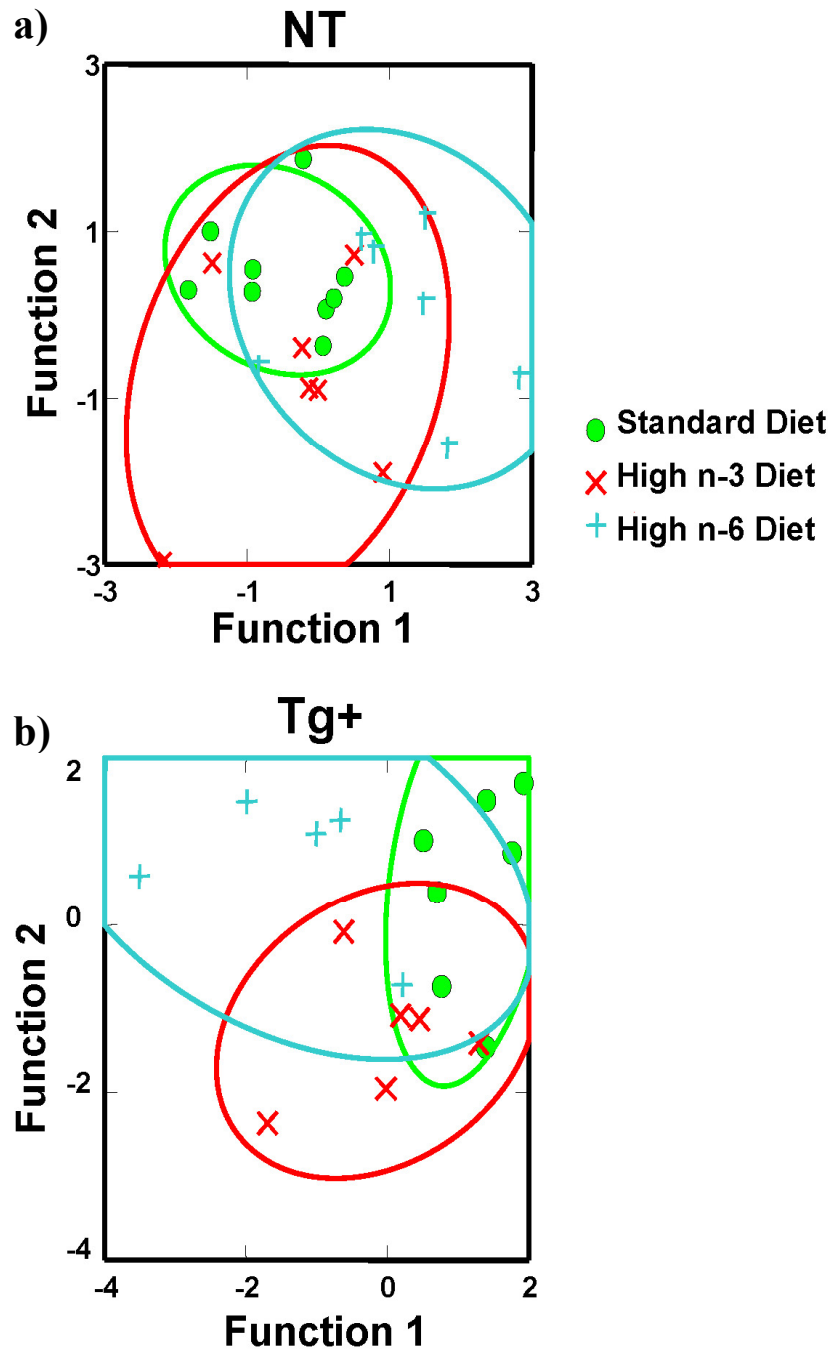


Fig. 45. Canonical scores plots of direct entry DFA's for the three groups of NT mice (a) and the three groups of Tg+ mice (b). The eight cognitive-based measures that loaded on Factor 1 of Factor Analysis were utilized. The DFA could not successfully discriminate between any of the three dietary groups within the NT animals. Similarly, none of the three Tg+ groups could be discriminated from the others based on their overall cognitive performance across the 8 cognitive measures utilized.

Table 13. Summary of discriminant function analyses including NT and Tg+ mice.

| Measures | Groups | Direct Entry Method (All Measures Used) | Stepwise-Forward Method Significance | Measures Retained |
|---------------------------------------|--------|--|---|--|
| All 19 | NT | N.S. | p < 0.05 *A vs C *B vs C | CPE-Fin |
| | Tg+ | N.S. | p < 0.05 | RAWM-T5-B3 RAWM-T5 WM-Ret PR-Fin PR-Avg CPE-Avg CPE-Fin YM-Alt EP-#C |
| Factor 1 (7 cognitive measures) | NT | N.S. | N.S. | |
| | Tg+ | N.S. | p < 0.02 *A vs C *B vs C | RAWM-T5-B3 PR-Fin |

Notes:

(1) p-values are from Wilks's lambda.

(2) Post-hoc analysis revealed complete discrimination between groups, except where otherwise indicated. * Group codings: A = standard diet, B = high n-3 diet, C = high n-6 diet

Abbreviation: N.S. = not significant; YM-Alt = Y-maze % alternation; all other abbreviations defined in Table 10.

Table 14. Summary of discriminant function analyses including NT and APP/PS1 mice for the standard and high n-3 groups.

| Measures | Groups | Direct Entry Method (All Measures Used) | Stepwise-Forward Method Significance | Measures Retained |
|---------------------------|---------|--|---|--|
| All 19 | NT | N.S. | N.S. | |
| | APP/PS1 | N.S. | p < 0.0005 | RAWMT4 CPE-Fin YM-Alt EP-#O EP-#C STR |
| Factor 1 | NT | N.S. | N.S. | |
| (9 cognitive measures) | APP/PS1 | N.S. | p < 0.05 | RAWMT5 T5-B3 |

Notes:

(1) p-values are from Wilks's lambda.

(2) Post-hoc analysis revealed complete discrimination between groups, except where otherwise indicated.

Abbreviation: N.S. = not significant; YM-Alt = Y-maze % alternation; STR = string agility; all other abbreviations defined in Table 10.

Discussion

In the present work, we evaluated the behavioral and pathological effects of active A β immunotherapy and dietary administration of omega-3 and omega-6 fatty acids in APP/PS1 and NT mice. Previous work has shown the significance of using transgenic animals to test various therapeutics for AD, such as environmental enrichment, dietary supplementations or vaccination protocols. Within the present work, there was a greater cognitive benefit from A β vaccination as compared to omega-3 dietary intervention. However, neither study resulted in any significant alteration of A β levels, indicating that any cognitive benefits observed through either A β immunotherapy or dietary fatty acid administration were A β independent.

I. A β Vaccination Discussion

Immunotherapeutic approaches to AD first began in the 1996 with *in vitro* work using monoclonal antibodies against A β (Solomon et al., 1996). It wasn't until 1999, however, that the first study was performed on an AD transgenic mouse model (Schenk et al., 1999). Within the last 6 years, many studies have focused on the use of either active or passive vaccinations in transgenic mice; however, few have behaviorally assessed the efficacy of such immunotherapy. The current study tested the effects of life-long active A β vaccination on behavioral and A β deposition measures in AD transgenic

mice. Following initiation of immunotherapy at 2 months of age, these mice were behaviorally evaluated at an early time point (4½-6 months) and a late time point (15-16½months) in an extensive battery that analyzed multiple sensorimotor and cognitive domains. Thus Tg+ were vaccinated over a 14½ month period (essentially most of their adult life) to determine if life-long vaccinations protect against cognitive impairment in both adulthood and old age. This study represents the longest period of time any AD vaccination protocol has utilized in any species. Briefly, this protracted protocol of immunotherapy protected AD transgenic mice from impairment in a variety of cognitive measures that were otherwise present at both test points. This improvement in cognition, however, occurred without any change in compact A β deposition within the brains of these mice. Nevertheless, factor analysis revealed an underlying relationship between compact A β and multiple cognitive measures, indicating an interaction between brain A β and cognitive performance.

Behavioral Effects - Sensorimotor and Anxiety-based Tasks

Within this study, at the early time point, the Tg+/A β group exhibited increased activity over multiple measures. The increased activity could be due to higher levels of soluble A β or an interaction of the A β /antibody complex within the hippocampus, which has been shown to be influential on locomotor activity (Laghmouch et al., 1997). Alternatively, the increased activity could be due to glutamate activation by A β ₁₋₄₂, resulting in over-activation of NMDA receptors (Brunskill et al., 2005). Over activation of NMDA receptors in the hippocampus has previously been shown to result in increased motor activity (Rogers et al., 1989).

Subjects with AD have been shown to exhibit reduced anxiety levels (Gauthier, 1998; Stout et al., 2003), as did the Tg+/C mice at the late test point. However, because the Tg+/A β mice had corrected their level of anxiety to the level of the NT mice, the A β vaccination corrected their disinhibition. Comparable to findings by Arendash et al. (2001), the Tg+/C group at either test point had similar overall activity to the NT mice. In addition, both balance beam and string agility tasks did not result in any difference between the Tg+/C mice and the NT mice. This indicates that any cognitive impairment in the Tg+/C mice was not due to sensorimotor impairments.

Behavioral Effects - Cognitive-based Tasks

Similar to the balance beam and string agility tasks, both Y-maze and circular platform tasks did not reveal any difference in performance between the Tg+/Con and NT groups at either test point. Therefore, there was no potential for the immunotherapy to correct the performance within these tasks. These findings are similar to those by Arendash et al. (2001a) and King and Arendash (2002) that found no change in performance between NT and APP Tg+ mice at similar ages for the Y-maze task. Importantly, measures from both Y-maze and circular platform tasks loaded independent from other cognitive-based measures in the factor analysis, as our laboratory has previously reported (Leighty et al., 2004). This implies that these two tasks measure separate cognitive domains as compared to the water-based memory tasks.

Most active vaccination studies that reported behavioral findings only used single tasks to identify any cognitive improvement. Janus et al. (2000) reported that 17 weeks of A β ₁₋₄₂ injections to 15 week old TgCRND8 mice resulted in improved spatial

reference learning, compared to control mice. Similarly, the present study also found a significant improvement at a young age in AD transgenic mice due to repeated vaccinations (see below). However at the aged test point, the results were less impressive, possibly due to the loss of Tg⁺ mice between the two test points, resulting in lower group size and consequently less power for statistical analysis. It is noteworthy that the survival rate of APP/PS1 mice in the present study was comparable to the survival rate of APP mice reported by King and Arendash (2002). Nevertheless, at both test points, Tg⁺/Con mice were impaired versus NT mice in spatial reference learning during Morris maze testing. In contrast, Arendash et al. (2001a) found that young adult APP/PS1 mice were not impaired in spatial reference learning in Morris water maze. A possible explanation for the discrepancy could be the slight alteration in A β processing/aggregation after generations of crossbreeding, thereby making the APP transgene more sensitive to the cognitive domain required for Morris water maze acquisition. At both young and aged timepoints, A β immunotherapy was effective at protecting against reference learning impairment, particularly at the earlier test point, as evidenced by Morris water maze acquisition results. Similar to Morris water maze acquisition, A β immunotherapy protected against the memory retention deficit otherwise present at the young time point, as shown in the Morris water maze probe trial. However, by the late time point, the immunotherapy was unable to protect against the continued memory impairment exhibited by these same mice. The lack of effect of the vaccination could be partially due to the reduced number of animals in both Tg⁺ control and treatment groups. Alternatively, it may be due to the high plaque load these animals have accumulated by 16 months of age, which was not prevented by A β immunotherapy and

that adversely affected their memory retention. In contrast, Kotilinek et al. (2002) reported an improvement in both Morris water maze acquisition and retention memory in adult Tg2576 mice following 1 week of passive immunization. However, the results indicated a significant treatment effect only for pre-treatment versus post-treatment testing of the Tg+ mice and not for a direct comparison of post-treatment performance of Tg+ controls vs. Tg+/A β treated mice.

The platform recognition task, performed immediately following Morris water maze, measured the animals' ability to switch strategies. During Morris water maze, the mice were required to use a spatial strategy to find a submerged platform; however, the platform recognition task required the animals to use a different strategy - identification/recognition learning. At the early time point, no effect of the A β immunotherapy could be seen because all groups performed well. At the aged test point, however, the performance of Tg+/C mice was impaired vs. NT controls. However, the performance of A β -treated Tg+ mice was no different from the NT mice or from any of the groups at the early test point, indicating that A β immunotherapy protected these mice from impairment in switching strategies. This immunization-induced protection is especially relevant to patients with AD, who commonly have deficits in attention-shifting tasks (Amieva et al., 2004; Tales et al., 2004; Dorion et al., 2002). Deficits in attention-shifting tasks have been shown to be regionalized in the parietal cortex (Buck et al., 1997). Adult Tg+ mice show A β plaques in the cortex (Dodart et al., 2000b), which could contribute to the impairment in strategy-switching of the Tg+/C mice. Therefore, it is evident that A β immunotherapy administered early in life and continued as a prophylactic can protect AD Tg+ mice from impairment in strategy switching.

Previously, the radial arm water maze (RAWM) task has been shown to be sensitive to working memory impairment and A β deposition (Arendash et al., 2001a; Gordon et al., 2001; Nilsson et al., 2004; Leighty et al., 2004). By the last block of the task, and for both time points, the present study's Tg+/C mice exhibited poor performance. This is in conjunction with short-term memory impairments, which progress throughout the disease process in patients with AD. In contrast, A β immunotherapy improved the performance of Tg+ at both ages such that the Tg+/A β mice were no different from NT mice by the last block of testing. Morgan et al. (2000) had similar results after 8 months of A β ₁₋₄₂ injections, such that A β -treated mice performed significantly better than control Tg+ mice and no different from NT mice. Likewise, Sigurdsson et al. (2003) injected 6-8 month old Tg2576 mice with A β ₁₋₃₀ for 13 months and found an overall significant improvement in working memory performance in an 8-arm radial maze. However, the use of A β immunotherapy in aged APP/PS1 mice did not reverse any of the working memory impairments exhibited in RAWM testing, as reported by Austin et al. (2003). In that later study, the lack of a treatment effect could be due to the much shorter period of vaccination and/or the much greater A β burdens that the "aged" APP/PS1 had when A β immunotherapy was begun. Results from the present study indicate that long-term A β immunotherapy is effective at protecting against working memory impairments in AD Tg+ mice if implemented at a young or adult age. Summarizing the behavioral data in this study, A β immunotherapy administered for 15 months effectively protected AD Tg+ mice against cognitive deficits

in spatial learning/reference memory (Morris water maze), strategy switching/recognition (platform recognition), and working memory (RAWM).

A β Histopathology

Surprisingly, all of the cognitive improvements previously reported occurred without any change in compact A β deposition. Therefore, all of the cognitive benefits from life-long A β immunotherapy were independent of alterations of A β deposition. Several prior vaccination studies have also shown cognitive benefits of immunotherapy in behavioral tasks without concurrent changes in A β measures. Morgan et al. (2000) only found a reduction in A β in the cortex, not hippocampus, despite significant improvements in working memory. In addition, passive immunotherapy has been reported to result in improvements in cognition with no alterations in amyloid deposition. Regarding the latter, Dodart et al. (2002) found that administration of m266 (specific to A β_{13-28}) improved object recognition memory after a single injection, with no concurrent change in A β deposition. The authors suggested that the behavioral benefit from the vaccination is more likely due to peripheral clearance or sequestration of soluble A β , and not by altering deposited forms of A β within the brain. In effect, the present study and others suggest that behavioral improvements resulting from A β immunotherapy are possibly due to neutralization of soluble A β oligomers from the brain. This effect occurs early in A β aggregation, prior to manifest A β fibrillar formation, such that improvements in cognition could occur at an early age, prior to overt A β deposition, as was seen the present study for the early time point.

Correlations and Multimetric Analyses

Previous work has demonstrated significant correlations between cognitive measures and A β deposition; however, the present study found no such correlations. Most notably this could be due to the lack of change in A β deposition between the transgenic groups, despite the improved cognitive performance of Tg+/A β mice. Also, because previous work from the same mouse line (Arendash et al., 2001; Gordon et al., 2001) found significant correlations between cognitive performance and A β pathology measures, generations of crossbreeding for the present study could have affected the behavioral phenotype of the transgenic mice, as well as the fact that the present study involved a full test battery (run twice). Despite the lack of correlations, FA analysis revealed significant underlying relationships between multiple cognitive measures and A β deposition. In prior work from our laboratory, factor analysis has been successfully used in transgenic AD mouse models to identify underlying relationships between cognitive and pathological measures (Leighty et al., 2004). Studies that employ a comprehensive test battery that encompasses multiple cognitive and sensorimotor domains have the advantage of utilizing multimetric analyses, such as FA and DFA, in order to characterize and distinguish between different treatment groups based on their performances. Leighty et al. (2004) demonstrated significant associations between cognitive performance and A β deposition measures within four separate PDAPP mouse lines. At both the early and late test point the current study found one primary factor (factor 1) that was largely cognitively based and one factor (factor 2) that loaded mainly sensorimotor measures. At the late test point, both with and without A β pathologic measure, one measure of elevated plus maze (EP-TO) loaded separately in its own factor.

While the remaining two factors (EP-#C and EP-#O) loaded with activity or exploratory measures, the “percent time in open arms” clearly measures more of a true anxiety component. Also at the late test point, A β pathologic measures loaded primarily within factor 1, which remained cognitively-based. However, the findings from factor 2 associated cognitive and sensorimotor components in addition to one measure of A β ; whereas without pathologic measures, factor 2 did not include any cognitive measures. This indicates that A β interacts with measures of activity in addition to measures of cognition.

In addition to FA, discriminant function analysis (DFA) has also been used successfully in transgenic mouse models studies. This analysis attempts to discriminate between groups of mice based on their performance. Within the present study, although the “direct entry” method was relatively ineffective in discriminating between the 3 groups, the DFA step-wise forward method was very effective at distinguishing between the NT and Tg⁺ groups. At the later test point, starting with all 19 behavioral measures, this DFA was able to discriminate between the Tg⁺ groups. This indicates that even after 15 months of active A β immunizations, A β vaccinated Tg⁺ were still clearly distinguishable from Tg⁺ controls based on their sensorimotor measures and cognitive performance. In addition, a stepwise forward DFA using only the top cognitive measures from factor 1 significantly separated the NT and Tg⁺/Con groups, but could not distinguish cognitive performance of the NT and Tg⁺/A β groups. The measures retained for providing the discrimination between NT and Tg⁺/Con mice included two measures of RAWM, and one from platform recognition, indicating that the transgenic behavioral

effect at 15-16½ months is largely based on working memory and recognition/identification.

In summary, life-long active A β immunotherapy was effective at protecting AD Tg+ mice from impairment in a variety of cognitive domains . This protection provided to Tg+ mice extended across all of the water-based cognitive tasks and well into older age. Since this cognitive protection occurred without a decrease in A β deposition, the mostly likely mechanism of A β immunotherapy's action involves neutralization/removal of small A β oligomers from the brain.

Because of the effectiveness of several A β immunotherapy studies in AD transgenic models of Alzheimer's disease, clinical trials were performed. A Zurich cohort of AD subjects that had received active vaccinations of A β_{1-42} showed stabilization of memory and preserved hippocampal function in conjunction with the production of antibodies (Hock, 2003). Also, antibodies from these inoculated AD subjects were reactive to A β plaques from AD Tg+ mouse brains and A β from human brains (Hock, 2002). However, 5 % of a separate cohort of AD subjects that were injected with A β_{1-42} developed a severe inflammatory response (e.g., meningoencephalitis) to the vaccine or to the vaccine's adjuvant. Therefore, these trials were halted and less risky approaches for AD immunotherapy are currently being explored. Alternative vaccination therapies which might offer less negative side effects include passive vaccinations, immunization with partial A β fragments, or injection of T-cells.

II. Omega-3 Discussion

Numerous epidemiologic studies suggest that a higher intake of fish oils is associated with a reduced risk of dementia later in life (Kalmijn et al., 1997a; Kalmijn et al., 1997b, Kalmijn et al., 2004). This reduced risk is thought to be more specifically contributed by the n-3 fatty acids, DHA and/or EPA, which are highly concentrated in fish (Kalmijn et al., 1997a; Kalmijn et al., 1997b). Despite this notion, there have been no long-term clinical studies that have administered either DHA or EPA to subjects with dementia as a treatment, or prior to onset of cognitive impairment in the form of a prevention-based study. In addition, the majority of rodent studies that have utilized fish oils or DHA supplementation have largely first involved DHA or n-3 depletion prior to supplementation (Gamoh et al., 2001; Ikemoto et al., 2001; Calon et al., 2004; Hashimoto et al., 2005). In non-transgenic rodents, there was no cognitive benefit to supplementation with DHA after multiple generations of n-3 deficiency (Gamoh et al., 2001; Ikemoto et al., 2001). Likewise, Calon et al. (2004) found no benefit of recognition/identification memory or memory retention in either non-transgenic or APP transgenic mice supplemented with DHA. However, APP mice enriched with DHA showed an improvement in memory acquisition in the Morris water maze (Calon et al., 2004). In addition, Hashimoto et al. (2005) showed an improvement in working and reference memory in rats supplemented with DHA, after being fed a fish oil deficient diet for 3 generations and infused with A β ₁₋₄₀. Thus, the effects of n-3 fatty acid supplementation in rodents is equivocal, with the later two studies related to Alzheimer's

disease not designed to address the central question of whether n-3 fatty acids are cognitively protective against AD.

The major aims of the present study were to: 1) determine the cognitive effects of a high n-3 diet in both NT and AD transgenic mice, 2) determine the cognitive effects of a typical American diet (high in n-6 and low in n-3 fatty acids) in both NT and AD transgenic mice, and 3) determine the effects of these two diets on blood cytokine levels, brain fatty acid levels, and brain A β levels (Tg+ mice only). The high n-3 diet mimicked humans having fish twice weekly, with a dietary n-6/n-3 ratio of 4/1. In essence, this study investigated whether an n-3 enriched diet, begun in young adulthood, can protect against inevitable cognitive impairment in AD transgenic mice and whether a high n-6 enriched diet with an n-6/n-3 ratio of 50/1 can contribute to greater cognitive impairment in AD transgenic mice. Moreover, since both diets were also given to non-transgenic mice, cognitive effects in normal animals could also be tested.

Survival Analysis.

Prior to an assessment of the behavioral impact of a high n-3 or high n-6 diet in Tg+ mice (see below), a study was performed to determine the effects of an n-3 deficient diet on survivability. Briefly, this study found that an interaction between the background and transgene profile determined the survivability of mice that were fed an n-3 deficient diet. F1 generation PS1 mice had increased survival on an n-3 deficient diet as compared to F1 APP mice and F2 generation APP, PS1 and NT mice. The F1 generation had a relatively high amount of B6 background (25%), which primarily contributed to the increased survival of the PS1 mice. Mice with 100% B6 background

have been shown to be less sensitive to dietary alterations in fat, and have minimal changes in metabolism when shifting between high fat to low fat diets (Biddinger et al., 2005). In addition, mice with a high B6 background also have elevated expression of fatty acid synthase (FAS), malic enzyme and δ -5 desaturase, - all of which convert available fatty acids into long-chain fatty acids (Coleman, 1992; Biddinger et al., 2005). FAS is critical in *de novo* lipogenesis to ultimately contribute to the production of palmitate. Palmitate is a precursor to downstream production of long-chain fatty acids. Malic enzyme also contributes to the *de novo* production of palmitate. Thus, the B6 background appears to have contributed to increased lipid metabolism in order to compensate for the n-3 deficiency and reduced n-6 dietary content. A combination of the increased expression of FAS, malic enzyme and δ -5 desaturase due to the increased B6 background from the F1 versus the F2 generation clearly contributed to the increased survivability of PS1 mice on a diet devoid of n-3 fatty acids.

As to why the PS1 transgene contributed to survival whereas the APP^{sw} transgene did not, there is no clear association between either of these transgenes and lipid metabolism. Previous work has shown that the presence of a mutant APP transgene in mice leads to increased inflammation, oxidative damage and dystrophic neurites (Irizarry et al., 1997; Benzing et al., 1999; Melhorn et al., 2000; Pratico et al., 2001). These factors alone are not detrimental to the survival of APP mice at their young age. However, the combination of the severe reduction in n-6 fatty acids, the lack of n-3 fatty acids in the omega-3 deficient diet, and the presence of mutant APP could account for their low survival. In the present study, the F2 generation mice had a reduced percentage of B6 background (12.5%), which was insufficient to contribute to survival irrespective

of genotype. In summary, the higher B6 background and PS1 genotype of F1 generation mice was able to compensate for the a diet low in n-6 and deficient in n-3 fatty acids.

Behavioral, Pathologic and Neurochemical Assessments.

Briefly, this study determined that in NT mice, long-term n-3 supplementation did not provide any cognitive benefits. Similarly, in Tg⁺ mice, n-3 supplementation largely resulted in cognitive performance that was no better than that of mice fed a standard diet or a diet high in n-6 fatty acids. For both NT and Tg⁺ mice, the high n-6 diet did not result in an overall worse cognitive performance as compared to the standard-fed mice. These results suggest that DHA and EPA supplementation may only provide limited, or no cognitive protection against AD. It is possible, however, that dietary fish contains additional beneficial nutrients, besides DHA and EPA, that could offer protection against development of AD in humans. Additionally, there were no major diet-induced changes in plasma cytokine levels or brain A β deposition (the later for Tg⁺ mice). However NT mice did show some marked diet-induced changes in cortical fatty acids levels. Within the high n-6 fed NT mice, most of the n-6 fatty acids were elevated as compared to the high n-3 fed NT mice. Similarly, half of the n-3 fatty acids were elevated in the high n-3 fed NT mice as compared to the high n-6 fed NT mice. However, in the APP/PS1 mice, there were minimal diet-induced changes in cortical fatty acid levels with either a high n-3 or high n-6 diet. Additionally, there were no diet-induced changes in soluble or insoluble hippocampal A β or plasma cytokines between any of the three APP/PS1 groups. Despite the minimal changes in cognitive and pathologic measures, multivariate analyses revealed significant separations between the dietary groups of the Tg⁺ and

more specifically between the standard and high n-3 APP/PS1 mice. However, within the NT mice, only the high n-6 fed mice were discriminated from both the standard and high n-3 NT mice.

Behavioral Effects - Sensorimotor and Anxiety-based Tasks

Prior rodent studies that have evaluated sensorimotor and anxiety effects from fish oil supplementation found both an increase (Carrie et al., 2000) and decrease (Chalon et al., 1998) in locomotor activity; however no significant changes in anxiety-based tasks (Chalon et al., 1998; de Wilde et al., 2002). The present study did not reveal any differences in sensorimotor/anxiety measures among the three dietary groups of NT mice. Thus, no effects of high n-3 or high n-6 diets were evident on sensorimotor function and anxiety level in normal mice. These results are in contrast to several prior reports involving normal rodents. Carrie et al. (2000) showed increased locomotor activity in mice that were supplemented with fish oil. However, Chalon et al. (1998) found reduced activity in young rats that had been supplemented with fish oil for two generations. Chalon et al. (1998) also measured anxiety in the elevated plus maze task and found no effect with fish oil supplementation. In conjunction with this, de Wilde et al. (2002) also found no difference in elevated plus maze measures with n-3 PUFA enrichment in rats. The results from prior studies are therefore inconsistent, perhaps reflecting different methodologies. In contrast to the lack of dietary effects on sensorimotor function/anxiety in the present study's NT mice, Tg⁺ mice given n-3 supplementation had increased activity in two separate tasks (open field and Y-maze). The reason(s) for why an

increased level of activity results from n-3 supplementation in Tg+ mice, but not NT mice, are unknown and would be based on conjecture without further study.

No previous studies have evaluated the effects of n-3 or n-6 supplementation on balance beam or string agility in either normal or Alzheimer's transgenic mice. For both NT and Tg+ mice of the present study, there were no effects of either the n-3 or the n-6 diet on either of these tasks. These results underscore that neither diet had deleterious effects on sensorimotor function and that any dietary effects on cognitive-based tasks within the NT or Tg+ groups could not be attributable to sensorimotor impairment.

Behavioral Effects - Cognitive-based Tasks

Previous work involving alterations of dietary n-3 or n-6 fatty acids evaluated cognitive performance primarily in the Morris water maze task (Jensen et al., 1996; Wainwright et al., 1999; Carrie et al., 2000; de Wilde et al., 2002; Barcelo-Coblijn et al., 2003; Calon et al., 2004). A few studies (all from the same group) have reported enhanced performance with n-3 supplementation in maze learning using a simple maze construction with only one entry/exit and many blind alleys (Suzuki et al., 1998; Lim & Suzuki, 1999; Lim & Suzuki, 2000). One study that utilized the Tg2576 APP transgenic mouse model found marginal improvement in spatial memory learning from Morris water maze acquisition with DHA enrichment (Calon et al., 2004). It is important to underscore, however, that the Calon et al. (2004) study involved DHA supplementation to Tg2576 mice that were previously on a DHA-deficient diet. As mentioned earlier, this experimental model has marginal relevance to human-related studies. Within the present study there were minimal effects of either diet across a variety of cognitive measures in

NT or Tg⁺ mice. The few effects of high n-3 or high n-6 diets can primarily be summarized as follows: n-6 enrichment in Tg⁺ and NT mice resulted in impaired spatial learning/memory in the circular platform task as compared to standard-fed groups within their genotypes, n-6 supplementation in Tg⁺ mice resulted in improved memory retention as seen in the Morris water maze, and n-3 supplementation in APP/PS1 mice resulted in improvement in recognition/identification in the platform recognition task, as compared to the standard-fed APP/PS1 mice.

Because there was no difference in Y-maze percent alternation between the standard Tg⁺ mice and the standard NT mice, no improvement due to fatty acid administration could be seen in the Tg⁺ mice. Also, in the NT mice, supplementation of n-3 fatty acids did not result in any improvement in alternation performance; as well, supplementation of n-6 fatty acids did not result in a lower percent alternation in either Tg⁺ or NT mice. Even though no other previous work has evaluated the effects of n-3 or n-6 fatty acid supplementation on spatial learning/memory in the circular platform task, the present study showed that dietary enrichment of either NT or Tg⁺ mice with n-6 fatty acids resulted in cognitive impairment in this task. Although the circular platform task measures spatial reference learning and memory similar to Morris water maze, circular platform measures loaded on factors independent of other cognitive measures in factor analysis, indicating a separate cognitive domain used in this task as compared to Morris maze and water-based cognitive tasks in general. Clearly, circular platform uses unique cognitive domains that were sensitive to n-6 supplementation in both Tg⁺ and NT mice.

Likewise to the above-mentioned tasks, the standard-fed Tg⁺ mice performed similar to the standard-fed NT mice in Morris water maze. Despite this lack of a

transgenic impairment, there was ample opportunity for the n-3 supplementation to improve upon Morris maze performance in Tg+ mice, and yet no such improvement occurred with n-3 supplementation. The lack of a transgenic impairment in Morris acquisition is in opposition to the impairment seen in 5½ month old APP mice (Arendash et al., 2004) and 4½-6 month old APP/PS1 mice previously reported in this dissertation (Jensen et al., 2005). The lack of an improvement or impairment in Morris maze acquisition with either n-3 or n-6 dietary intervention in NT mice is consistent with all other NT rodent studies that have used Morris water maze to evaluate cognitive effects of fatty acid supplementation. Jensen et al. (1996) found no improvement in spatial learning/memory after 4 generations of fish oil supplementation in either acquisition or retention memory. Additional work involving supplementation of young rats with DHA or fish oil did not result in improved spatial memory (Wainwright et al., 1999; Barcelo-Coblijn et al., 2003). Also, de Wilde et al. (2002) showed that adult rats did not behaviorally benefit from n-3 supplementation in Morris water maze acquisition or retention. Thus, the present study's lack of any n-3 dietary effect on Morris maze acquisition in normal rodents is consistent with the prior literature.

Within Tg2576 APP mice Calon et al (2004) reported that DHA enrichment to an n-3 deficient diet resulted in improved acquisition in Morris water maze. However, the improvement in acquisition was only noted in the latter phase of Morris maze acquisition. Thus, there was no effect overall of transgenicity, DHA enrichment or low dietary DHA. The design of the Calon study done in AD Tg+ mice and the present study's design are not comparable. Nonetheless, both studies found no effects of a high n-3 diet on Morris maze retention – this is, if all 6 groups of the present study are included in the analysis.

If only APP/PS1 mice are compared for a high n-3 vs. standard diet, however, the present study found that n-3 supplemented Tg+ mice showed a partial improvement in memory retention. Surprisingly, the high n-6 Tg+ mice showed a complete restoration in memory retention to the same level as the NT mice. Within the NT mice, opposite results were seen, such that the high n-6 mice showed impairment in memory retention. Thus, the same diet involving high n-6 fatty acids had opposite effects in NT and Tg+ mice. Several previous studies have shown that NT mice supplemented with n-3 fatty acids show no benefit in Morris water maze acquisition or retention (Jensen et al., 1996; Wainwright et al., 1999; Carrie et al., 2000), while NT mice fed low dietary n-3 fatty acids are not impaired in Morris water maze (Calon et al., 2004). Since no prior Morris maze studies involved administration of a high n-6 diet to AD Tg+ mice, there is a lack of information in the available literature that could account for the discrepancy between the response of NT and Tg+ to a high n-6 diet.

The platform recognition task requires that mice switch strategies from the spatial memory of Morris water maze to a search/identification strategy. Calon et al. (2004) found no effect of dietary DHA in NT mice in this task, similar to the results from the present study. However, Calon et al. (2004) also found no difference overall between APP and NT mice, nor with the addition of DHA to either genotype. However, the present study found a clear impairment of Tg+ mice fed a high n-6 diet as compared to mice fed a high n-3 fatty acids. Although, n-3 fatty acids have been shown to be important in visual development (Marin et al., 2000), this is most likely not the reason for the impairment of high n-6 Tg+ mice. All Tg+ and NT groups performed similar in MWM acquisition in which they were required to use spatial cues, illustrating that all

groups had similar visual abilities. Thus, there was a clear cognitive protection offered by n-3 fatty acids to AD Tg+ mice compared to the impairment otherwise evident in Tg+ mice fed a diet high in n-6 fatty acids, independent of visual abilities. These results are quite significant in relation to subjects with AD that have difficulty in strategy switching or attention shifting, as previously discussed in a prior section.

The radial arm water maze (RAWM) task has been shown to be a sensitive index of working memory and also sensitive to hippocampal A β deposition (Arendash et al., 2001; Gordon et al., 2001; Nilsson et al., 2004; Leighty et al., 2004). To date, there have been no other studies that evaluated the effects of either omega-3 or omega-6 fatty acid supplementation to NT or AD Tg+ mice on radial arm water maze (RAWM) working memory performance. However, Sugimoto et al., (2002) found that adult mice that were supplemented with DHA had improved working memory, as indexed by the 8-arm radial maze task. In contrast, Gamoh et al. (2001) found no improvement in working memory in the same task from aged rats that were given dietary DHA after 3 generations on a fish oil deficient diet. Similarly, the present study illustrated that there were no, or minimal effects of diet in either NT or Tg+ mice on RAWM working memory performance.

Although, both high n-3 and high n-6 diet Tg+ mice performed similar to standard NT mice by the last block of testing, neither diet group provided any significant improvement or impairment in memory as compared to the standard Tg+ mice. This indicates that n-6 enrichment did not result in a greater deficit as compared to either a standard or high n-3 diet in either NT or Tg+ mice. Thus, high dietary n-6 fatty acids in normal individuals, or those predisposed to AD would not appear to induce an increased risk of developing a working memory deficit. In addition, the current study showed that n-3 supplementation

was not able correct working memory impairments in Tg⁺ mice and provided essentially no benefit to working memory in Tg⁺ mice.

A β Pathology

Puskas et al. (2003) and Barcelo-Coblijn et al. (2003) found that fish oil supplementation to rats increased expression of transthyretin (TTR). TTR has been previously shown to solubilize fibril A β and inhibit aggregation of A β *in vitro* (Schwarzman & Goldgaber, 1996; Redondo et al., 2000). TTR has the potential to both inhibit initial formation of A β deposition and clear compact amyloid from the brains of rodents fed fish oil. However, the present study found no alterations of either soluble or insoluble hippocampal A β ₁₋₄₀ or A β ₁₋₄₂ levels from APP/PS1 mice fed a high n-3 diet. Also, there were no differences in hippocampal soluble or insoluble A β ₁₋₄₀ or A β ₁₋₄₂ in high n-6 APP/PS1 mice compared to either the high n-3 or standard-fed APP/PS1 mice. In contrast, Lim et al. (2005) found that DHA enrichment to aged AD Tg⁺ mice previously fed an n-3 deficient diet reduced total insoluble A β in addition to total (insoluble + soluble) A β ₄₂ and total A β ₄₀ in the cortex. Despite the altered A β levels, TTR expression was unchanged in those mice, indicating an alternative mechanism by which DHA altered amyloid levels.

Cytokine Levels

Previous works shows conflicting results indicating both pro-inflammatory and anti-inflammatory expression resulting from n-3 supplementation. Billiar et al. (1988) and Renier et al. (1993) showed that macrophages from rodents fed dietary fish oil

resulted in an anti-inflammatory effect. By contrast, Watanabe et al. (1993) and Petursdottir et al. (2002) showed that rodents fed a diet enriched with fish oil had increased pro-inflammatory cytokines from peritoneal macrophages. The present study found no significant differences in plasma cytokine levels of NT mice fed a high n-3 vs. high n-6 diet. These results are in contrast to all four of the above studies, perhaps in part due to their use of macrophages to measure cytokines rather than the current study's use of plasma; alternatively their treatment groups were compared to standard diet animals, in contrast to the n-3 vs. n-6 group comparisons. For the most part, human studies have supported anti-inflammatory actions of n-3 fatty acids. Blok et al. (1996) reviewed many of the human studies involving n-3 fatty acid with inflammatory diseases. To summarize his review, administration of dietary n-3 fatty acids resulted in improvements in rheumatoid arthritis, Systemic Lupus Erythematosus (SLE), psoriasis and ulcerative colitis. The primary mechanism to reduce inflammation is through EPA metabolism. EPA, from fish oil, rapidly incorporates into cell membranes and replaces AA in the phospholipids. This replacement increases the production of a less active prostaglandin (E3) and leukotriene (B5), thereby reducing the inflammatory response.

In the present study, plasma cytokine levels from Tg⁺ mice indicated a trend toward reduction in all cytokines (pro- and anti-inflammatory) in plasma for Tg⁺ mice fed a high n-6 diet vs. a high n-3 diet. Therefore, the present study supports both pro- and anti-inflammatory enhancement in Alzheimer's Tg⁺ mice fed a high n-6 diet.

Brain Fatty Acid Levels

AD subjects have been found to have a reduced amount of EPA, DHA and total n-3 fatty acids in brain tissue as compared to elderly control subjects (Corrigan et al., 1998; Conquer et al., 2000). This reduction in n-3 PUFAs as compared to control elderly persons further provides evidence that the pathology associated with AD is independent of the normal aging process. Although the cause of the alterations in fatty acid content in the brain of AD patients is unknown, some studies suggest that there is an alteration in fatty acid metabolism by the liver, increased oxidative damage, or a reduction in dietary intake of specific fatty acids (Soderberg, et al., 1991; Skinner et al., 1993; Corrigan et al., 1998; Prasad et al., 1998). Fatty acid metabolism proceeds primarily such that dietary fatty acids are metabolized by the liver and transported via the blood to target organs, such as the brain. The present study found that alterations in brain fatty acids were affected by both the AD genotype and dietary intake. However, all three diets (standard, high n-3 and high n-6) contained similar amounts of saturated and monounsaturated fatty acids. As such, there were no significant differences in cortical saturated or monounsaturated fats within the three NT or three APP/PS1 groups. There were also no differences between the NT or APP/PS1 mice on any of the three diets, indicating that there is no difference in metabolism of saturated or monounsaturated fats in APP/PS1 mice as compared to NT mice.

For the NT mice fed a high n-6 diet, many of the cortical n-6 fatty acids (including total n-6 fatty acids) were elevated versus the high n-3 fed mice. Likewise for the NT mice fed a high n-3 diet, half of the n-3 fatty acids were elevated versus the high n-6 NT mice. Thus, in the NT mice, the frontal cortex content of fatty acids is reflective

of their dietary intake. However, despite the significant changes in both n-3 and n-6 cortical levels, minimal changes in behavioral performance were seen between the NT groups. These results provide further evidence for a lack of relationship between DHA and/or EPA intake and cognitive function. Within APP/PS1 mice, only one n-6 fatty acid (22:5n-6) was elevated in the high n-6 group versus the high n-3 group. Also, there were no differences in cortical n-3 content between any of the three APP/PS1 groups. Therefore, unlike the NT mice, despite the significant difference in dietary intake of n-3 and n-6 fatty acids, the concurrent changes in brain fatty acid levels were not evident.

In contrast to the current study, Calon et al. (2005) found significant decreases in frontal cortex n-6 fatty acids and increases in n-3 fatty acids in aged Tg2576 APP mice fed a low n-3 diet supplemented with DHA. The same results were also seen in aged NT mice on a low n-3 diet that was also supplemented with DHA. The authors suggested that the difference in frontal cortex fatty acid levels of the low n-3 fed APP mice was most likely due to the initial n-3 depletion in combination with the presence of the APP transgene. Calon et al. (2005) suggested that for the low n-3 group, the APP transgene provided oxidative stress that rapidly depleted the brain of DHA. However in a direct comparison between the genotypes, the present study found that among the high n-6 mice, there were minimal differences in brain fatty acid levels when comparing NT to APP/PS1 mice. However, the current study also found significant increases in several n-6 fatty acids within the APP/PS1 high n-3 mice versus the high n-3 NT mice. Thus, despite identical dietary intake, APP/PS1 mice show a deficit in metabolizing polyunsaturated fatty acids for incorporation into brain tissue as compared to NT mice. Therefore enhanced dietary intake of n-3 or n-6 fatty acid in normal mice resulted in

elevated brain levels of n-3 and n-6 fatty acids, respectively; however, these same elevations were not seen in APP/PS1 mice.

Correlations

Two sets of correlations were performed involving NT mice 1) for all three diet groups or 2) for only the high n-3 and high n-6 groups. Briefly, these correlations revealed a positive relationship between saturated, monounsaturated, n-3 and n-6 fatty acids in the frontal cortex and cognitive impairment in multiple tasks, primarily RAWM and platform recognition. These correlations show that brain total fatty acid levels, irrespective of their form, are indicative of cognitive impairment. However, Greenwood and Winocur (1996) showed that learning impairment is directly related to intake of saturated fats, and independent of monounsaturated or polyunsaturated fatty acid intakes. More recently, Winocur and Greenwood (2005) showed that rats fed a diet high in saturated fats were impaired on memory and learning tasks. Likewise, Morris et al. (2005) found that increased intake of n-6 and n-3 fatty acids in addition to saturated fats plays an important role in cognitive function and risk of dementia. Similar to the present study, Suzuki et al., (1998) found that mice that had increased n-6 fatty acids in the brain stem were poorer performers as compared to mice that had increased n-3 content in the brain stem. However, the authors did not perform any direct correlations. Similarly, Ikemoto et al. (2001) also showed that an increase in brain n-6 fatty acid levels is related to learning impairment in a brightness-discrimination task.

Similar to NT mice, correlations among APP/PS1 mice revealed a significant relationship between cortical n-6 levels and cognitive impairment in multiple tasks.

Previous work has also shown that elevated levels of brain n-6 fatty acids are associated with impaired learning or memory in AD Tg+ mice (Calon et al., 2004) or AD-like rats (Hashimoto et al., 2002). Studies of human AD subjects also show a relation between a reduction in brain n-6 fatty acids and reduced prevalence of dementia in AD (Corrigan et al., 1998; Prasad et al., 1998). Prasad et al. (1998) not only showed a correlation between AD and reduction of brain n-6 fatty acids, but also an association of β -amyloid plaques in the regions that had reduced n-6 fatty acid levels. Likewise, the present study showed a significant correlation between hippocampal A β levels and cognitive impairment. This is in agreement with previous work showing multiple correlations between brain A β deposition and a variety of cognitive measures (Arendash et al., 2001a; Gordon et al., 2001; Leighty et al., 2004). Lastly, both cortical n-3 and n-6 fatty acid levels in frontal cortex were overall correlated with plasma cytokines – higher brain levels of n-3 and n-6 fatty acids were associated with lower levels of pro-inflammatory cytokines (e.g., an anti-inflammatory effect). As mentioned in one of the below sections, the relation between polyunsaturated fatty acids and cytokine levels is conflicting, as supported by the correlation results.

FA and DFA

In agreement with the A β vaccination study of this dissertation and previous studies from our laboratory, the factor analysis results involving behavioral measures from the present study show that factor 1 is loaded exclusively by cognitive measures, whereas factor 2 is sensorimotor/anxiety-based (Leighty et al., 2004; Jensen et al., 2005). Whether or not APPsw mice were included with the APP/PS1 mice in the factor analysis,

the factor loadings remained relatively similar. Inclusion of neurochemical measures showed associations between multiple cognitive domains and both A β measures and n-6 fatty acids. In support, similar associations were also seen within several correlations as previously discussed.

In addition to FA, a number of discriminant function analyses (DFAs) were performed in order to determine if the separate dietary groups within each genotype (Tg+ or NT) could be significantly distinguished based on their sensorimotor and cognitive performances. Direct entry DFA was completely unsuccessful at discriminating between any of the dietary groups of NT or Tg+ (with or without APP mice) mice. However, the step-wise forward DFA could discriminate between the high n-6 NT group and both the standard and high n-3 NT groups using all measures (sensorimotor and cognitive). In addition, step-wise forward DFA was ineffective at discriminating between the three NT groups using only the top seven cognitive measures from the FA factor 1, or the two NT groups (standard and high n-3) using either all measures or the top nine cognitive measures from FA factor 1. This indicates that the fatty acid diets partially contributed to the overall performance of the NT mice, despite the lack of significant difference between these groups on any given task. However, there were several significant differences in cortical fatty acid levels within the separate NT groups, which, according to the correlation results were significantly associated with multiple cognitive domains. Clearly, the cortical fatty acid content in NT mice has an underlying relationship to cognitive performance.

The step-wise forward DFA's for Tg+ and APP/PS1 mice were more effective at discriminating between dietary groups than the DFA's for the NT mice. Step-wise

forward DFA was successfully used to completely separate all three Tg⁺ groups and both standard and high n-3 APP/PS1 groups using all 19 behavioral measures. This indicates an overall contribution of dietary fatty acids to performance on all tasks collectively (sensorimotor, anxiety, and cognitive) in Tg⁺ mice despite a lack of effect on individual tasks between the 3 Tg⁺ dietary treatment groups. Also, Tg⁺ mice fed the high n-6 diet were able to be separated from both the standard and high n-3 Tg⁺ mice with the step-wise forward DFA using only the top seven cognitive measures from the FA factor 1. This is the same separation seen from the step-wise forward DFA using all measures in NT mice. Lastly, step-wise forward DFA completely discriminated standard-fed APP/PS1 mice from high n-3-fed APP/PS1 mice using their top nine cognitive measures from the FA factor 1. Thus, despite a lack of difference in many of the behavioral tasks, n-3 fatty acid supplementation contributed significantly to the overall behavioral performance of APP/PS1 mice. Taken together with the correlation results and FA measures, it is evident that dietary intake of fatty acids are closely associated with multiple cognitive domains in both NT and Tg⁺ mice. Although none of the direct entry DFA's were successful, all of the step-wise forward DFA's were completely successful at separating the high n-3 and high n-6 diet groups for Tg⁺ mice (with or without APP mice). Overall, this indicates that the increase in n-3 fatty acids in the high n-3 diet was sufficient to alter the behavior of AD Tg⁺ mice such that they could be separated based solely on their behavioral performances.

Clinical Implications of Omega-3 Fatty Acids for Prevention Against AD

Within NT mice, there was no overall cognitive benefit of dietary n-3 supplementation. Conversely, there were no overall impairments due to the n-6 enrichment. Thus, the high n-6 content of a typical American diet does not by itself lead to cognitive impairment in individuals not pre-disposed to AD. However, since a high n-6 fatty acid diet is typically linked with a diet high in saturated fatty acids, it is more likely that the saturated fats in a typical American diet increase the risk of cognitive impairment. Supportive of this premise, Morris et al. (2005) found little evidence for an association between fish intake and rate of cognitive decline. Instead, Morris found that the positive relationship between fish intake and cognitive protection was most significant when including ALA supplementation over a long-term period, thus the protection was not solely due to the fish intake. In addition, rodents that have been fed a high fat diet are clearly impaired in spatial learning in the Morris water maze (Zhao et al., 2004) and learning/memory in the variable-interval delayed attention task (Winocur & Greenwood, 1996; Greenwood & Winocur, 1999), underscoring the increased risk of cognitive impairment induced by a high fat diet.

Although in the AD transgenic mice, there were limited cognitive benefits of high n-3 fatty acid supplementation compared to a standard diet, a high n-3 diet did not result in significantly better cognitive performance in any task compared to a high n-6 diet. Indeed, Tg⁺ mice on a high n-6 diet were not worse overall on any cognitive task compared to those on a high n-3 diet. Therefore, even in subjects predisposed to AD, a diet high in n-6 fatty acids does not necessarily lead to increased cognitive impairment. Rather, it would appear that the high saturated fat content often associated with a high n-6

diet is the determining factor in increased risk of AD for a typical American diet. Along this line, Morris et al. (2005) proposed that overall fat consumption had a higher link to cognitive decline and Alzheimer's disease than a diet simply low in n-3 fatty acids. The present work suggests that DHA, EPA, and/or fish oil supplements may provide only limited, or no cognitive protection against AD, as well as no reduction in A β levels in the brain. Also, the protective effects of a high fish diet, as shown in previous epidemiological studies, may involve other beneficial nutrients in fish besides a high content of n-3 fatty acids – nutrients that could confirm some protection against AD-related dementia. Alternatively, the postulated protection of a high fish diet could be related to the otherwise healthful lifestyle adopted by many fish eater (e.g., a lower fat diet, high intake of fruits and vegetables, increased exercise).

IV. Overall Conclusions

Life-long active vaccination and dietary omega-3 fatty acids to AD Tg⁺ mice resulted in two different levels of cognitive protection, with A β vaccinations clearly being more beneficial. There was a more extensive and consistent long-term cognitive protection offered by the A β immunotherapy across multiple cognitive domains, whereas long-term omega-3 supplementation results in very limited cognitive benefit in selected cognitive measures. Both long-term treatments resulted in no change in brain A β levels, indicating that any cognitive benefits were independent of either A β deposition (A β immunotherapy study) or A β generation (dietary study) in the brain. This suggests that the cognitive protection from the A β vaccine may be due to neutralization of soluble

oligomers or a completely A β -independent mechanism. Nonetheless, the multimetric analyses for both studies showed a clear association/relationship between brain A β levels and cognitive factors. Because there were no differences in plasma cytokine levels in the n-3 dietary study, any cognitive protection offered by omega-3 supplementation was independent of a “global” inflammatory response, although the possibility of a more localized “brain” inflammatory response cannot be eliminated because brain levels of cytokines were not evaluated. Nonetheless, there were numerous alterations of cortical fatty acid levels that significantly correlated with several behavioral tasks. Thus, there is a clear link between brain fatty acid levels and cognitive function, which was also underscored by the factor analysis. Lastly, both studies approached AD therapeutics from a prevention-based arena in utilizing Alzheimer’s Tg⁺ mice to determine the long-term effects of active A β immunotherapy and dietary fatty acid manipulation in highly controlled prospective studies where all other variables were controlled for. Since such longitudinal protective-based studies in humans are impractical and cannot be tightly controlled, testing various therapeutic and/or prophylactic interventions in AD transgenic models represents a critically important venue for developing effective interventions against Alzheimer’s Disease.

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About the Author

Maren Jensen received her Bachelor's Degree in Biology from the University of North Florida in 2000. She entered the Ph.D. program in Biology at the University of South Florida in 2002. Her work in the field has been highlighted in a co-authored publication as well as by a platform presentation at the Society for Neuroscience meeting. While in the Ph.D. program, Ms. Jensen worked as a researcher for the Johnnie B. Byrd Alzheimer's Center and Research Institute.